

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
24 December 2003 (24.12.2003)

PCT

(10) International Publication Number
WO 03/106633 A2

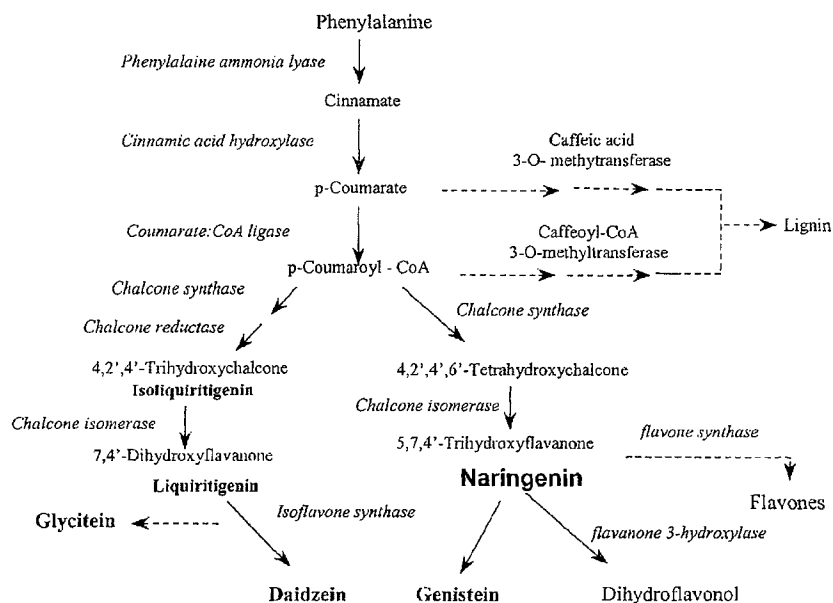
- (51) International Patent Classification⁷: C12N
- (21) International Application Number: PCT/US03/18663
- (22) International Filing Date: 12 June 2003 (12.06.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
60/388,280 13 June 2002 (13.06.2002) US
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- (81) Designated States (national): AF, AG, AI, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii)) for the following designations AF, AG, AI, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK,

[Continued on next page]

(54) Title: METHOD TO INCREASE ISOFLAVONOID LEVELS IN PLANTS AND PLANTS PRODUCING INCREASED LEVELS OF ISOFLAVONOID



(57) Abstract: This invention pertains to methods of increasing isoflavonoid production in isoflavonoid-producing plants by transforming plants with at least one construct expressing at least a portion of a flavanone 3-hydroxylase, a C1 myb transcription factor, and an R-type myc transcription factor that regulate expression of genes in the phenylpropanoid pathway.



MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, UZ, VC, VN, YU, ZA, ZM, ZW, ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SI, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG)

- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii)) for all designations

— of inventorship (Rule 4.17(iv)) for US only

Published:

- without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

TITLEMETHOD TO INCREASE ISOFLAVONOID LEVELS IN PLANTS
AND PLANTS PRODUCING INCREASED LEVELS OF ISOFLAVONOIDS

This application claims the benefit of U.S. Provisional Application
5 No. 60/388,280, filed 13 June 2002. The entire content of this provisional
application is herein incorporated by reference.

This invention pertains to methods of increasing isoflavonoid production in
isoflavonoid-producing plants by transforming plants with at least one construct
comprising nucleic acid fragments encoding at least a portion of a flavanone
10 3-hydroxylase, a C1 myb transcription factor, and an R-type myc transcription factor.

Isoflavonoids represent a class of secondary metabolites produced in legumes
by a branch of the phenylpropanoid pathway and include such compounds as
isoflavones, isoflavanones, rotenoids, pterocarpans, isoflavans, quinone derivatives,
3-aryl-4-hydroxycoumarins, 3-arylcoumarins, isoflav-3-enes, coumestans,
15 alpha-methyldeoxybenzoin, 2-arylbenzofurans, isoflavanol, coumaronochromone
and the like. In plants, these compounds are known to be involved in interactions
with other organisms and to participate in the defense responses of legumes against
phytopathogenic microorganisms (Dewick, P. M. (1993) in *The Flavonoids*,
Advances in Research Since 1986, Harborne, J. B. Ed., pp. 117-238, Chapman and
20 Hall, London). Isoflavonoid-derived compounds also are involved in symbiotic
relationships between roots and rhizobial bacteria which eventually result in
nodulation and nitrogen-fixation (Phillips, D. A. (1992) in *Recent Advances in*
Phytochemistry. Vol. 26, pp 201-231, Stafford, H. A. and Ibrahim, R. K., Eds,
Plenum Press, New York), and overall they have been shown to act as antibiotics,
25 repellents, attractants, and signal compounds (Barz, W. and Welle, R. (1992)
Phenolic Metabolism in Plants, pg 139-164, Ed by H. A. Stafford and R. K. Ibrahim,
Plenum Press, New York).

Isoflavonoids have also been reported to have physiological activity in animal
and human studies. For example, it has been reported that the isoflavones found in
30 soybean seeds possess antihemolytic (Naim, M., et al. (1976) *J. Agric. Food Chem.*
24:1174-1177), antifungal (Naim, M., et al. (1974) *J. Agr. Food Chem.* 22:806-810),
estrogenic (Price, K. R. and Fenwick, G. R. (1985) *Food Addit. Contam.* 2:73-106),
tumor-suppressing (Messina, M. and Barnes, S. (1991) *J. Natl. Cancer Inst.*
83:541-546; Peterson, G., et al. (1991) *Biochem. Biophys. Res. Commun.*
35 179:661-667), hypolipidemic (Mathur, K., et. al. (1964) *J. Nutr.* 84:201-204), and
serum cholesterol-lowering (Sharma, R. D. (1979) *Lipids* 14:535-540) effects. In
addition, both epidemiological and dietary-intervention studies indicate that when

isoflavones in soybean seeds and in subsequent protein products prepared from the seeds are part of the human dietary intake, those products provide many significant health benefits (Messina, M. J. (1999) *Am. J. Clin. Nutr.* 70:439S-450S).

Free isoflavones rarely accumulate to high levels in soybeans. Instead they
5 are usually conjugated to carbohydrates or organic acids. Soybean seeds contain three types of isoflavones aglycones, glucosides, and malonylglucosides. Each isoflavone type is found in three different forms: daidzein, genistein, and glycitein form the aglycones; daidzin, genistin, and glycitin form the glucosides; and 6"-O-malonyldaidzin, 6"-O-malonylgenistin and 6"-O-malonylglycitin form the
10 malonylglucosides. During processing acetylglucoside forms are produced: 6'-O-acetyldaidzin, 6'-O-acetyl genistin, and 6'-O-acetyl glycitin. The content of isoflavonoids in soybean seeds is quite variable and is affected by both genetics and environmental conditions such as growing location and temperature during seed fill (Tsukamoto, C., et al. (1995) *J. Agric. Food Chem.* 43:1184-1192; Wang, H. and Murphy, P. A. (1994) *J. Agric. Food Chem.* 42:1674-1677). In addition,
15 isoflavonoid content in legumes can be stress-induced by pathogen attack, wounding, high UV light exposure and pollution (Dixon, R. A. and Paiva, N. L. (1995) *Plant Cell* 7:1085-1097). The genistein isoflavonoid forms make up the most abundant group in soybean seeds and most food products, while the daidzein and the glycitein forms are present in lower levels (Murphy, P.A. (1999) *J. Agric. Food Chem.* 47:2697-2704).

The biosynthetic pathway for isoflavonoids in soybean and their relationship with several other classes of phenylpropanoids is presented in Figure 1. The
25 enzyme flavanone 3-hydroxylase (F3H; EC 1.14.11.9) catalyzes the conversion of flavanones to dihydroflavonols, which are intermediates in the biosynthesis of flavonols, anthocyanidins, catechins and proanthocyanidins. This enzyme is also referred to as naringenin 3-dioxygenase, and naringenin, 2-oxoglutarate 3-dioxygenase, among others. In soybean, both flavanone 3-hydroxylase and isoflavone synthase (IFS) compete for naringenin as a substrate and it is not clear
30 how this competition is regulated. Suppression of F3H has been shown to increase the resistance of cultivated plants to chemical stress (PCT publication WO 00/78979, published December 28, 2000).

Though the branch initiated by isoflavone synthase that leads to synthesis of isoflavonoids is mainly limited to the legumes, the remainder of the phenylpropanoid
35 pathway occurs in other plant species. In maize, genes of the phenylpropanoid pathway are regulated by the transcription factor C1 in combination with an R-type factor. Together C1 and an R-type factor activate expression of a set of genes that

leads to the synthesis and accumulation of anthocyanins in maize cells (Grotewold, E., et al. (1998) *Plant Cell* 10:721-740).

Maize C1 is a myb-type transcription factor that regulates expression of genes involved in anthocyanin production and accumulation in maize cells. However C1
5 cannot activate gene expression alone, and requires interaction with an R-type myc transcription factor for activation of target gene promoters. The R-type factors include, among others, alleles of R, alleles of the homologous B gene of maize, and alleles of the homologous Lc gene. These genes function similarly and make up the R/B gene family (Goff, S.A., et al. (1992) *Genes Dev.* 6:864-875). The various
10 genes of the R/B gene family may be in turn each found as diverging alleles that fluctuate in expression pattern within the corn plant due to differences in their promoters. The members of this family encode proteins with very similar amino acid sequences and thus have comparable effects on the anthocyanin pathway structural genes. The specificity of the different promoters provides tissue
15 specificity of anthocyanin biosynthesis (Radicella, J.P. et al. (1992) *Genes Dev.* 6:2152-2164; Walker, E.L. (1995) *EMBO J.* 14:2350-2363). The skilled artisan will recognize that the coding region of any functional gene of this large family could be used in conjunction with a promoter of choice to obtain R-gene function in the desired tissue or developmental stage. Examples of R/B family genes and alleles
20 include, but are not limited to, Lc, R, R-S, R-P, Sn, B-Peru, and B-I. The coding regions of particular alleles of the Lc or B genes, especially the B-Peru allele, have been most commonly used in experiments in conjunction with C1.

Cell suspension lines of the maize inbred Black Mexican Sweet (BMS) that were transformed with an estradiol-inducible version of a fusion of C1 and R (CRC)
25 were analyzed after the addition of estradiol. The cDNA fragments from the known flavonoid genes, except chalcone isomerase, were induced in the CRC-expressing line after hormone induction (Bruce et al. (2000) *Plant Cell* 12:65-80). Maize C1 and an R-type factor together can promote the synthesis of anthocyanins in *Arabidopsis* tissues that do not naturally express anthocyanins (Lloyd, A.M., et al.
30 (1992) *Science* 258:1773-1775), and in petunia leaves (Quattrocchio, F., et al. (1993) *Plant Cell* 5:1497-1512). WO 99/37794, published July 29, 1999, discloses that the expression of maize C1 and the Lc allele of R in tomato fruit results in increased levels of the flavonol kaempferol. Thus, it is known that C1 and an R-type factor can regulate expression of individual genes of the phenylpropanoid
35 pathway in plants including *Arabidopsis*, petunia, tomato, and maize leading to production of anthocyanins or flavonols. These are all plants that do not produce isoflavonoids. Isoflavonoid production is almost exclusively limited to the legumes.

An example of one of the few non-legume plants that does produce isoflavonoids is sugar beet.

- WO 00/44909, published August 3, 2000, discloses transformation of soybeans with maize C1 and R (as a CRC chimera) in conjunction with overexpression of the isoflavone synthase gene. Visual inspection, after transient expression of C1 and B-Peru in white clover and pea, which are legumes, shows production of anthocyanin in several tissues (Majnik, et al. (1998) *Aust. J. Plant Phys.* 25:335-343). Any possible effect of C1 and B-Peru on isoflavonoid levels was not assayed. Expression of a CRC chimera in an isoflavonoid-producing plant alters the levels of isoflavonoids in the transgenic plant. Soybean plants expressing the CRC chimera contain higher levels of daidzein and lower levels of genistein, with the total daidzein to total genistein ratios being increased, when compared to an untransformed plant (US patent application 60/297981 having Attorney Docket No. BB1452 US NA and cofiled with the present on June 13, 2002).
- The physiological benefits associated with isoflavonoids in both plants and humans make the manipulation of their contents in crop plants highly desirable. For example, increasing levels of isoflavonoids in soybean seeds increases the efficiency of extraction and lowers the cost of isoflavone-related products sold today for use in either reduction of serum cholesterol or in estrogen replacement therapy. Therefore there is a need to enhance the level of isoflavonoids in isoflavonoid-producing plants. Combining suppression of flavanone 3-hydroxylase (F3H) with activation of the phenylpropanoid pathway is one method to accomplish this goal.

SUMMARY OF THE INVENTION

- The invention concerns a method of increasing isoflavonoid production in an isoflavonoid-producing plant, the method comprising:
- a) transforming a plant with (1) a first recombinant DNA construct comprising a polynucleotide selected from the group consisting of: (i) a polynucleotide encoding all or part of a flavanone 3-hydroxylase; (ii) a polynucleotide from 5' and/or 3' ends of an isolated nucleic acid fragment which encodes flavanone 3-hydroxylase; or (iii) a polynucleotide comprising (i) and (ii) ; and (2) at least one second recombinant DNA construct comprising a polynucleotide encoding a C1 myb transcription factor and a R myc-type transcription factor; and b) growing the transformed plant of (a); and c) evaluating the plant or plant part obtained from the transformed plant for an increased quantity of isoflavonoid in the transformed plant or plant part as compared plant or plant parts obtained from an untransformed plant.

In a second embodiment, the first recombinant DNA construct described above comprises a promoter operably linked, in a sense or anti-sense orientation, to a polynucleotide encoding all or part of a flavanone 3-hydroxylase, or a promoter operably linked to a stem-loop structure selected from the group consisting of: (1) a structure wherein the loop comprises the polynucleotide nucleotide encoding all or part of a flavanone 3-hydroxylase and (2) a structure wherein the stem comprises the polynucleotide nucleotide encoding all or part of a flavanone 3-hydroxylase.

In a third embodiment, the isoflavonoid-producing plant is selected from the group consisting of soybean, clover, mung bean, lentil, hairy vetch, alfalfa, lupine, sugar beet, and snow pea. Also of interest are seeds or plant parts of a plant transformed with a recombinant expression construct of the invention from which isoflavonoid-containing products can be obtained or extracted.

In a fourth embodiment, this invention concerns a food or beverage incorporating these isoflavonoid-containing products.

In a fifth embodiment, this invention concerns a method of producing an isoflavonoid-containing product which comprises: (a) cracking the seeds obtained from plants transformed with any of the recombinant expression constructs of the invention to remove the meats from the hulls; and (b) flaking the meats obtained in step (a) to obtain the desired flake thickness.

In a sixth embodiment, the invention concerns an isoflavonoid-producing plant comprising in its genome:

(a) a first recombinant DNA construct comprising a polynucleotide selected from the group consisting of: (i) a polynucleotide encoding all or part of a flavanone 3-hydroxylase; (ii) a polynucleotide from 5' and/or 3' ends of an isolated nucleic acid fragment which encodes flavanone 3-hydroxylase; or (iii) a polynucleotide comprising (i) and (ii) ; and

(b) at least one second recombinant DNA construct comprising a polynucleotide encoding a C1 myb transcription factor and a R myc-type transcription factor; wherein seeds obtained from the transformed plant have an increased quantity of isoflavonoid as compared to seeds obtained from an untransformed plant.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE LISTINGS

The invention can be more fully understood from the following detailed description and the accompanying Sequence Listing which form a part of this application.

Figure 1 depicts the soybean biosynthetic pathway from phenylalanine to glycitein, daidzein, genistein, and dihydroflavonol.

Figure 2 depicts a representation of the seed-specific expression vector pKS151. The two copies of the 36-nucleotide sequence are indicated by "EL", and the inverted repeat of the 36-nucleotide sequence are indicated by "(EL)".

Figure 3 depicts the total isoflavones as well as the genistin, daidzin, and glycitin levels observed for individual R1 seeds from transformed plants expressing the F3H construct.

Figure 4 through Figure 9 depict the total isoflavones as well as the genistin, daidzin, and glycitin levels observed for individual R1 seeds from transformed plants expressing the F3H construct together with the CRC chimeric construct.

Figure 10 depicts the total isoflavones as well as the genistin, daidzin, and glycitin levels observed for individual R2 seeds from transformed plants expressing the F3H construct together with the CRC chimeric construct.

The following sequence descriptions and Sequences Listing attached hereto comply with the rules governing nucleotide and/or amino acid sequence disclosures in patent applications as set forth in 37 C.F.R. §1.821-1.825. The Sequence Listing contains the one letter code for nucleotide sequence characters and the three letter codes for amino acids as defined in conformity with the IUPAC-IUB standards described in *Nucleic Acids Research* 13:3021-3030 (1985) and in the *Biochemical Journal* 219 (No. 2):345-373 (1984) which are herein incorporated by reference. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

SEQ ID NO:1 is the nucleotide sequence of plasmid pKS151 used for the expression of a fragment of flavanone 3-hydroxylase.

SEQ ID NO:2 is the nucleotide sequence of primer flavanone 3-hydroxylase NotI sense, used to amplify a flavanone 3-hydroxylase fragment from clone sfl1.pk0040.g11.

SEQ ID NO:3 is the nucleotide sequence of primer flavanone 3-hydroxylase NotI antisense, used to amplify a flavanone 3-hydroxylase fragment from clone sfl1.pk0040.g11.

SEQ ID NO:4 is the nucleotide sequence of the cDNA insert in plasmid sfl1.pk0040.g11, encoding a flavanone 3-hydroxylase.

SEQ ID NO:5 is the nucleotide sequence of primer 3, used to detect the presence of the flavanone 3-hydroxylase construct in transformed plants.

SEQ ID NO:6 is the nucleotide sequence of primer 4, used to detect the presence of the flavanone 3-hydroxylase construct in transformed plants.

SEQ ID NO:7 is the nucleotide sequence of primer 5, used to detect the presence of the flavanone 3-hydroxylase construct in transformed plants.

SEQ ID NO:8 is the nucleotide sequence of primer 6, used to detect the presence of the flavanone 3-hydroxylase construct in tranformed plants.

SEQ ID NO:9 is the nucleotide sequence of primer 1, used to detect the presence of the CRC chimeric construct in tranformed plants.

5 SEQ ID NO:10 is the nucleotide sequence of primer 2, used to detect the presence of the CRC chimeric construct in tranformed plants.

DETAILED DESCRIPTION OF THE INVENTION

All patents, patent applications and publications cited are incorporated herein by reference in their entirety.

10 In the context of this disclosure, a number of terms shall be utilized.

The term "isoflavonoid(s)" refers to a large group of polyphenolic compounds, based on a common diphenylpropane skeleton, which occur naturally in plants. This term, as used herein, includes, but is not limited to, the three types of isoflavones in three different forms: the aglycones, daidzein, genistein and glycitein; 15 the glucosides, daidzin, genistin and glycitin; and the malonylglucosides, 6"-O-malonyldaidzin, 6"-O-malonylgenistin and 6"-O-malonylglycitin, as well as, the acetylglucoside forms: 6'-O-acetyldaidzin, 6'-O-acetyl genistin, and 6'-O-acetyl glycitin that are formed during processing.

The term "isoflavonoid-producing plant" refers to a plant in which isoflavonoids 20 naturally occur. Examples of isoflavonoid-producing plants include, but are not limited to, soybean, clover, mung bean, lentil, hairy vetch, alfalfa, lupine, sugar beet, and snow pea. In a more preferred embodiment, the preferred isoflavonoid-producing plant would be soybean. Examples of other isoflavonoid-producing plants can be found in WO 93/23069, published November 25, 1993, the disclosure of 25 which is hereby incorporated by reference.

The term "flavanone 3-hydroxylase" refers to the polypeptide or enzyme that catalyzes the conversion of flavanones to dihydroflavonols.

The term "C1 myb transcription factor" refers to a protein encoded by a maize C1 gene and to any protein which is functionally equivalent to a C1 myb 30 transcription factor.

The term "R myc-type transcription factor" refers to a protein with a basic helix-loop-helix domain encoded by a member of the R/B gene family and to any protein that is functionally equivalent to an R myc-type transcription factor.

The terms "polynucleotide", "polynucleotide sequence", "nucleic acid 35 sequence", and "nucleic acid fragment"/"isolated nucleic acid fragment" are used interchangeably herein. These terms encompass nucleotide sequences and the like. A polynucleotide may be a polymer of RNA or DNA that is single- or double-

stranded, that optionally contains synthetic, non-natural or altered nucleotide bases. A polynucleotide in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA, synthetic DNA, or mixtures thereof. Nucleotides (usually found in their 5'-monophosphate form) are referred to by their single letter designation as follows: "A" for adenylate or deoxyadenylate (for RNA or DNA, respectively), "C" for cytidylate or deoxycytidylate, "G" for guanylate or deoxyguanylate, "U" for uridylate, "T" for deoxythymidylate, "R" for purines (A or G), "Y" for pyrimidines (C or T), "K" for G or T, "H" for A or C or T, "I" for inosine, and "N" for any nucleotide.

The terms "subfragment that is functionally equivalent" and "functionally equivalent subfragment" are used interchangeably herein. These terms refer to a portion or subsequence of an isolated nucleic acid fragment in which the ability to alter gene expression or produce a certain phenotype is retained whether or not the fragment or subfragment encodes an active enzyme. For example, the fragment or subfragment can be used in the design of chimeric genes to produce the desired phenotype in a transformed plant. Chimeric genes can be designed for use in suppression by linking a nucleic acid fragment or subfragment thereof, whether or not it encodes an active enzyme, in the sense or antisense orientation relative to a plant promoter sequence.

The terms "homology", "homologous", "substantially similar" and "corresponding substantially" are used interchangeably herein. They refer to nucleic acid fragments wherein changes in one or more nucleotide bases do not affect the ability of the nucleic acid fragment to mediate gene expression or produce a certain phenotype. These terms also refer to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not substantially alter the functional properties of the resulting nucleic acid fragment relative to the initial, unmodified fragment. It is therefore understood, as those skilled in the art will appreciate, that the invention encompasses more than the specific exemplary sequences.

Moreover, the skilled artisan recognizes that substantially similar nucleic acid sequences encompassed by this invention are also defined by their ability to hybridize, under moderately stringent conditions (for example, 0.5 X SSC, 0.1% SDS, 60°C) with the sequences exemplified herein, or to any portion of the nucleotide sequences disclosed herein and which are functionally equivalent to any of the nucleic acid sequences disclosed herein. Stringency conditions can be adjusted to screen for moderately similar fragments, such as homologous sequences from distantly related organisms, to highly similar fragments, such as

genes that duplicate functional enzymes from closely related organisms. Post-hybridization washes determine stringency conditions. One set of preferred conditions involves a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 min, then repeated with 2X SSC, 0.5% SDS at 45°C for 30 min, and then repeated twice with 0.2X SSC, 0.5% SDS at 50°C for 30 min. A more preferred set of stringent conditions involves the use of higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2X SSC, 0.5% SDS was increased to 60°C. Another preferred set of highly stringent conditions involves the use of two final washes in 0.1X SSC, 0.1% SDS at 65°C.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure. An "allele" is one of several alternative forms of a gene occupying a given locus on a chromosome. When all the alleles present at a given locus on a chromosome are the same that plant is homozygous at that locus. If the alleles present at a given locus on a chromosome differ that plant is heterozygous at that locus.

"Coding sequence" refers to a DNA sequence that codes for a specific amino acid sequence. "Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include, but are not limited to, promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

"Promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as

enhancers. Accordingly, an "enhancer" is a DNA sequence which can stimulate promoter activity, and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of some variation may have identical promoter activity. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro, J. K., and Goldberg, R. B. (1989) *Biochemistry of Plants* 15:1-82.

Among the most commonly used promoters are the nopaline synthase (NOS) promoter (Ebert et al. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:5745-5749), the octopine synthase (OCS) promoter, caulimovirus promoters such as the cauliflower mosaic virus (CaMV) 19S promoter (Lawton et al. (1987) *Plant Mol. Biol.* 9:315-324), the CaMV 35S promoter (Odell et al. (1985) *Nature* 313:810-812), and the figwort mosaic virus 35S promoter, the light inducible promoter from the small subunit of rubisco, the Adh promoter (Walker et al. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:6624-6628), the sucrose synthase promoter (Yang et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87:4144-4148), the R gene complex promoter (Chandler et al. (1989) *Plant Cell* 1:1175-1183), the chlorophyll a/b binding protein gene promoter, etc. Other commonly used promoters are, the promoters for the potato tuber ADPGPP genes, the sucrose synthase promoter, the granule bound starch synthase promoter, the glutelin gene promoter, the *maize* waxy promoter, Brittle gene promoter, and Shrunk 2 promoter, the acid chitinase gene promoter, and the zein gene promoters (15 kD, 16 kD, 19 kD, 22 kD, and 27 kD; Pedersen et al. (1982) *Cell* 29:1015-1026). A plethora of promoters is described in WO 00/18963, published on April 6, 2000, the disclosure of which is hereby incorporated by reference.

The "translation leader sequence" refers to a polynucleotide sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect processing

of the primary transcript to mRNA, mRNA stability or translation efficiency.

Examples of translation leader sequences have been described (Turner, R. and Foster, G. D. (1995) *Mol. Biotech.* 3:225-236).

5 The "3' non-coding sequences" refer to DNA sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht, I. L., et al.
10 (1989) *Plant Cell* 1:671-680.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript. An RNA transcript is referred to as the mature RNA when it is an RNA
15 sequence derived from post-transcriptional processing of the primary transcript. "Messenger RNA (mRNA)" refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a DNA that is complementary to and synthesized from a mRNA template using the enzyme reverse transcriptase. The cDNA can be single-stranded or converted into the double-stranded form using
20 the Klenow fragment of DNA polymerase I. "Sense" RNA refers to RNA transcript that includes the mRNA and can be translated into protein within a cell or *in vitro*. "Antisense RNA" refers to an RNA transcript that is complementary to all or part of a target primary transcript or mRNA, and that blocks the expression of a target gene (U.S. Patent No. 5,107,065). The complementarity of an antisense RNA may be
25 with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. "Functional RNA" refers to antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes. The terms "complement" and "reverse complement" are used interchangeably herein with respect to mRNA transcripts,
30 and are meant to define the antisense RNA of the message.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is regulated by the other. For example, a promoter is operably linked with a coding sequence when it is capable of regulating the expression of that coding sequence (i.e., that the coding
35 sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in a sense or antisense orientation. In another example, the complementary RNA regions of the invention can be

operably linked, either directly or indirectly, 5' to the target mRNA, or 3' to the target mRNA, or within the target mRNA, or a first complementary region is 5' and its complement is 3' to the target mRNA.

5 The term "expression", as used herein, refers to the production of a functional end-product e.g., a mRNA or a protein (precursor or mature).

"Mature" protein refers to a post-translationally processed polypeptide; i.e., one from which any pre- or propeptides present in the primary translation product have been removed. "Precursor" protein refers to the primary product of translation of mRNA; i.e., with pre- and propeptides still present. Pre- and propeptides may be
10 but are not limited to intracellular localization signals.

"Stable transformation" refers to the transfer of a nucleic acid fragment into a genome of a host organism, including both nuclear and organellar genomes, resulting in genetically stable inheritance. In contrast, "transient transformation" refers to the transfer of a nucleic acid fragment into the nucleus, or DNA-containing
15 organelle, of a host organism resulting in gene expression without integration or stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" organisms. Preferred methods of cell transformation for rice, corn, and other monocots are the particle-accelerated or "gene gun" transformation technology (Klein et al., (1987) *Nature (London)*
20 327:70-73; U.S. Patent No. 4,945,050), and an *Agrobacterium*-mediated method using an appropriate Ti plasmid containing the transgene (Ishida Y. et al., 1996, *Nature Biotech.* 14:745-750).

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook, J., Fritsch, E.F. and
25 Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989. Transformation methods are well known to those skilled in the art and are described above.

"PCR" or "Polymerase Chain Reaction" is a technique for the synthesis of large quantities of specific DNA segments, consists of a series of repetitive cycles
30 (Perkin Elmer Cetus Instruments, Norwalk, CT). Typically, the double stranded DNA is heat denatured, the two primers complementary to the 3' boundaries of the target segment are annealed at low temperature and then extended at an intermediate temperature. One set of these three consecutive steps is referred to as a cycle.

35 The term "recombinant" refers to an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis or by the

manipulation of isolated segments of nucleic acids by genetic engineering techniques.

The terms "recombinant construct", "expression construct", "chimeric construct", "construct" and "recombinant DNA construct" are used interchangeably herein. A recombinant construct comprises an artificial combination of nucleic acid fragments, e.g., regulatory and coding sequences that are not found together in nature. For example, a chimeric construct may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. Such construct may be used by itself or may be used in conjunction with a vector. If a vector is used then the choice of vector is dependent upon the method that will be used to transform host cells as is well known to those skilled in the art. For example, a plasmid vector can be used. The skilled artisan is well aware of the genetic elements that must be present on the vector in order to successfully transform, select and propagate host cells comprising any of the isolated nucleic acid fragments of the invention. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al., (1985) *EMBO J.* 4:2411-2418; De Almeida et al., (1989) *Mol. Gen. Genetics* 218:78-86), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, immunoblotting analysis of protein expression, or phenotypic analysis, among others.

"Antisense inhibition" refers to the production of antisense RNA transcripts capable of suppressing the expression of the target protein. "Co-suppression" refers to the production of sense RNA transcripts capable of suppressing the expression of identical or substantially similar foreign or endogenous genes (U.S. Patent No. 5,231,020). Co-suppression constructs in plants previously have been designed by focusing on overexpression of a nucleic acid sequence having homology to an endogenous mRNA, in the sense orientation, which results in the reduction of all RNA having homology to the overexpressed sequence (see Vaucheret et al. (1998) *Plant J* 16:651-659; and Gura (2000) *Nature* 404:804-808). The overall efficiency of this phenomenon is low, and the extent of the RNA reduction is widely variable. Recent work has described the use of "hairpin" structures that incorporate all, or part, of an mRNA encoding sequence in a complementary orientation that results in a potential "stem-loop" structure for the expressed RNA (PCT Publication WO 99/53050 published on October 21, 1999).

This increases the frequency of co-suppression in the recovered transgenic plants. Another variation describes the use of plant viral sequences to direct the suppression, or "silencing", of proximal mRNA encoding sequences (PCT Publication WO 98/36083 published on August 20, 1998). Both of these co-suppressing phenomena have not been elucidated mechanistically, although recent genetic evidence has begun to unravel this complex situation (Elmayan et al. (1998) *Plant Cell* 10:1747-1757).

The present invention concerns a method of increasing isoflavonoid production in an isoflavonoid-producing plant, said method comprising: a) transforming a plant with (1) a first recombinant DNA construct comprising a polynucleotide selected from the group consisting of: (i) a polynucleotide encoding all or part of a flavanone 3-hydroxylase; (ii) a polynucleotide from 5' and/or 3' ends of an isolated nucleic acid fragment which encodes flavanone 3-hydroxylase; or (iii) a polynucleotide comprising (i) and (ii); and (2) at least one second recombinant DNA construct comprising a polynucleotide encoding a C1 myb transcription factor and a R myc-type transcription factor; and b) growing the transformed plant of (a); and c) valuating the plant or plant parts obtained from the transformed plant for an increased quantity of isoflavonoid in the transformed plant or plant part as compared plant or plant part obtained from an untransformed plant.

The first recombinant DNA construct that may be used to transform plants of the present invention may comprise:

- (i) a polynucleotide encoding all or part of a flavanone 3-hydroxylase;
- (ii) a polynucleotide from 5' and/or 3' ends of an isolated nucleic acid fragment which encodes flavanone 3-hydroxylase; or
- (iii) a polynucleotide comprising (i) and (ii).

The polynucleotide mentioned above in (i), (ii), or (iii) may be operably linked to a promoter in a sense orientation or an antisense orientation, or may be forming part of a stem loop structure with the polynucleotide forming either the loop or the stem.

The second recombinant DNA construct, comprising a polynucleotide encoding a C1 myb transcription factor and an R myc-type transcription factor, which can be used to transform an isoflavonoid-producing plant, may comprise:

- (a) the polynucleotide encoding a C1 myb transcription factor and the polynucleotide encoding an R myc-type transcription factor can be entirely separate, e.g., one polynucleotide may comprise a promoter operably linked to an isolated nucleic acid fragment encoding a C1 myb transcription factor and another separate polynucleotide may comprise a promoter operably linked to an isolated nucleic acid fragment encoding an R-myc type transcription factor;

(b) the polynucleotide encoding a C1 myb transcription factor and the polynucleotide encoding an R myc-type transcription factor can be in a single construct comprising at least one promoter operably linked to an isolated nucleic acid fragment encoding a C1 myb transcription factor and at least one promoter operably linked to an isolated nucleic acid fragment encoding an R-myc type transcription factor; and

(c) the polynucleotide encoding a C1 myb transcription factor and the polynucleotide encoding an R myc-type transcription factor can be in a single construct comprising a promoter operably linked to an isolated nucleic acid fragment encoding all or a part of a C1 myb transcription factor and an isolated nucleic acid fragment encoding all or a part of an R-myc type transcription factor such that a fusion protein combining the two encoded proteins is produced.

The polynucleotide comprising a polynucleotide encoding at least a portion of a flavanone 3-hydroxylase and the polynucleotide comprising a C1 myb transcription factor and an R-myc type factor may each form part of at least one recombinant DNA construct, or may form part of one single recombinant DNA expression construct.

The transformed plant is then grown under conditions suitable for the expression of the recombinant DNA construct or constructs. Expression of the recombinant DNA construct or constructs alters the quantity of isoflavonoid of the transformed plant compared to the quantity of isoflavonoid of an untransformed plant.

In a more preferred, embodiment, an isoflavonoid-producing plant can be transformed with a recombinant expression construct comprising a promoter operably linked to a flavanone 3-hydroxylase and a recombinant expression construct comprising a promoter operably linked to an isolated nucleic acid fragment encoding a chimeric transcription factor comprising the maize R coding region situated between the C1 DNA binding domain and the C1 activation domain.

The regeneration, development and cultivation of plants from single plant protoplast transformants or from various transformed explants is well known in the art (Weissbach and Weissbach, In.: Methods for Plant Molecular Biology, (Eds.), Academic Press, Inc., San Diego, CA (1988)). This regeneration and growth process typically includes the steps of selection of transformed cells, culturing those individualized cells through the usual stages of embryonic development through the rooted plantlet stage. Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil.

The development or regeneration of plants containing the foreign, exogenous gene that encodes a protein of interest is well known in the art. Preferably, the regenerated plants are self-pollinated to provide homozygous transgenic plants. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important lines. Conversely, pollen from plants of these important lines is used to pollinate regenerated plants. A transgenic plant of the present invention containing a desired polypeptide is cultivated using methods well known to one skilled in the art.

There are a variety of methods for the regeneration of plants from plant tissue.

The particular method of regeneration will depend on the starting plant tissue and the particular plant species to be regenerated.

Methods for transforming dicots, primarily by use of *Agrobacterium tumefaciens*, and obtaining transgenic plants have been published for cotton (U.S. Patent No. 5,004,863, U.S. Patent No. 5,159,135, U.S. Patent No. 5,518, 908); soybean (U.S. Patent No. 5,569,834, U.S. Patent No. 5,416,011, McCabe et. al., *BiolTechnology* 6:923 (1988), Christou et al., *Plant Physiol.* 87:671-674 (1988)); *Brassica* (U.S. Patent No. 5,463,174); peanut (Cheng et al., *Plant Cell Rep.* 15:653-657 (1996), McKently et al., *Plant Cell Rep.* 14:699-703 (1995)); papaya; and pea (Grant et al., *Plant Cell Rep.* 15:254-258, (1995)).

Assays for gene expression based on the transient expression of cloned nucleic acid constructs have been developed by introducing the nucleic acid molecules into plant cells by polyethylene glycol treatment, electroporation, or particle bombardment (Marcotte et al., *Nature* 335:454-457 (1988); Marcotte et al., *Plant Cell* 1:523-532 (1989); McCarty et al., *Cell* 66:895-905 (1991); Hattori et al., *Genes Dev.* 6:609-618 (1992); Goff et al., *EMBO J.* 9:2517-2522 (1990)).

Transient expression systems may be used to functionally dissect gene constructs (see generally, Maliga et al., *Methods in Plant Molecular Biology*, Cold Spring Harbor Press (1995)). It is understood that any of the nucleic acid molecules of the present invention can be introduced into a plant cell in a permanent or transient manner in combination with other genetic elements such as vectors, promoters, enhancers etc.

In addition to the above discussed procedures, practitioners are familiar with the standard resource materials which describe specific conditions and procedures for the construction, manipulation and isolation of macromolecules (e.g., DNA molecules, plasmids, etc.), generation of recombinant DNA fragments and recombinant expression constructs and the screening and isolating of clones, (see for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring

Harbor Press (1989); Maliga et al., *Methods in Plant Molecular Biology*, Cold Spring Harbor Press (1995); Birren et al., *Genome Analysis: Detecting Genes*, 1, Cold Spring Harbor, New York (1998); Birren et al., *Genome Analysis: Analyzing DNA*, 2, Cold Spring Harbor, New York (1998); *Plant Molecular Biology: A Laboratory*
5 *Manual*, eds. Clark, Springer, New York (1997)).

Any promoter can be used in accordance with the method of the invention. Thus, the origin of the promoter chosen to drive expression of the coding sequence is not critical as long as it has sufficient transcriptional activity to accomplish the invention by expressing translatable mRNA for the desired nucleic acid fragments in
10 the desired host tissue. The promoter for use in the present invention may be selected from the group consisting of a seed-specific promoter, root-specific promoter, vacuole-specific promoter, and an embryo-specific promoter.

Examples of a seed-specific promoter include, but are not limited to, the promoter for β -conglycinin (Chen et al. (1989) *Dev. Genet.* 10: 112-122), the napin promoter, and the phaseolin promoter. Other tissue-specific promoters that may be
15 used to accomplish the invention include, but are not limited to, the chloroplast glutamine synthase (GS2) promoter (Edwards et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87:3459-3463), the chloroplast fructose-1,6-biophosphatase promoter (Lloyd et al. (1991) *Mol. Gen. Genet.* 225:209-2216), the nuclear photosynthetic (ST-LS1) promoter (Stockhaus et al. (1989) *EMBO J.* 8:2445-2451), the serine/threonine
20 kinase (PAL) promoter, the glucoamylase promoter, the promoters for the *Cab* genes (*cab6*, *cab-1*, and *cab-1R*, Yamamoto et al. (1994) *Plant Cell Physiol.* 35:773-778; Fejes et al. (1990) *Plant Mol. Biol.* 15:921-932; Lubberstedt et al. (1994) *Plant Physiol.* 104:997-1006; Luan et al. (1992) *Plant Cell* 4:971-981), the
25 pyruvate orthophosphate dikinase promoter (Matsuoka et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:9586-9590), the *LhcB* promoter (Cerdan et al. (1997) *Plant Mol. Biol.* 33:245-255), the *PsbP* promoter (Kretsch et al. (1995) *Plant Mol. Biol.* 28:219-229), the *SUC2* sucrose H^+ symporter promoter (Truernit et al. (1995) *Planta* 196:564-570), and the promoters for the thylakoid membrane genes (*psaD*,
30 *psaF*, *psaE*, *PC*, *FNR*, *atpC*, *atpD*), etc..

Also within the scope of this invention are seeds or plant parts obtained from such transformed plants. Plant parts include differentiated and undifferentiated tissues, including but not limited to, roots, stems, shoots, leaves, pollen, seeds, tumor tissue, and various forms of cells and culture such as single cells, protoplasts,
35 embryos, and callus tissue. The plant tissue may be in plant or in organ, tissue or cell culture.

In another aspect, this invention concerns an isoflavonoid-containing product high in total isoflavonoids obtained from the seeds or plant parts obtained from the transformed plants described herein. Examples of such an isoflavonoid-containing product include, but are not limited to, protein isolate, protein concentrate, meal, grits, full fat and defatted flours, textured proteins, textured flours, textured concentrates and textured isolates. In still another aspect, this invention concerns an isoflavonoid-containing product high in isoflavonoids extracted from the seeds or plant parts obtained from the transformed plants described herein. An extracted product could then be used in the production of pills, tablets, capsules or other similar dosage forms made to contain a high concentration of isoflavonoids.

Methods for obtaining such products are well known to those skilled in the art. For example, in the case of soybean, such products can be obtained in a variety of ways. Conditions typically used to prepare soy protein isolates have been described by [Cho, et al, (1981) U.S. Patent No. 4,278,597; Goodnight, et al. (1978) U.S. Patent No. 4,072,670]. Soy protein concentrates are produced by three basic processes: acid leaching (at about pH 4.5), extraction with alcohol (about 55-80%), and denaturing the protein with moist heat prior to extraction with water. Conditions typically used to prepare soy protein concentrates have been described by Pass [(1975) U.S. Patent No. 3,897,574] and Campbell et al. [(1985) in *New Protein Foods*, ed. by Altschul and Wilcke, Academic Press, Vol. 5, Chapter 10, *Seed Storage Proteins*, pp 302-338].

"Isoflavonoid-containing protein products" can be defined as those items produced from seed of a suitable plant which are used in feeds, foods and/or beverages. For example, "soy protein products" can include, but are not limited to, those items listed in Table 1. "Soy protein products".

TABLE 1

Soy Protein Products Derived from Soybean Seeds^a

<u>Whole Soybean Products</u>	<u>Processed Soy Protein Products</u>
Roasted Soybeans	Full Fat and Defatted Flours
Baked Soybeans	Soy Grits
Soy Sprouts	Soy Hypocotyls
Soy Milk	Soybean Meal
	Soy Milk
<u>Specialty Soy Foods/Ingredients</u>	Soy Protein Isolates
Soy Milk	Soy Protein Concentrates
Tofu	Textured Soy Proteins
Tempeh	Textured Flours and Concentrates
Miso	Textured Concentrates
Soy Sauce	Textured Isolates

Whole Soybean ProductsProcessed Soy Protein Products

Hydrolyzed Vegetable Protein

Whipping Protein

^aSee Soy Protein Products: Characteristics, Nutritional Aspects and Utilization (1987). Soy Protein Council.

"Processing" refers to any physical and chemical methods used to obtain the products listed in Table 1 and includes, but is not limited to, heat conditioning, flaking and grinding, extrusion, solvent extraction, or aqueous soaking and extraction of whole or partial seeds. Furthermore, "processing" includes the methods used to concentrate and isolate soy protein from whole or partial seeds, as well as the various traditional Oriental methods in preparing fermented soy food products. Trading Standards and Specifications have been established for many of these products (see National Oilseed Processors Association Yearbook and Trading Rules 1991-1992). Products referred to as being "high protein" or "low protein" are those as described by these Standard Specifications. "NSI" refers to the Nitrogen Solubility Index as defined by the American Oil Chemists' Society Method Ac4 41. "KOH Nitrogen Solubility" is an indicator of soybean meal quality and refers to the amount of nitrogen soluble in 0.036 M KOH under the conditions as described by Araba and Dale [(1990) *Poult. Sci.* 69:76-83]. "White" flakes refer to flaked, dehulled cotyledons that have been defatted and treated with controlled moist heat to have an NSI of about 85 to 90. This term can also refer to a flour with a similar NSI that has been ground to pass through a No. 100 U.S. Standard Screen size. "Cooked" refers to a soy protein product, typically a flour, with an NSI of about 20 to 60. "Toasted" refers to a soy protein product, typically a flour, with an NSI below 20. "Grits" refer to defatted, dehulled cotyledons having a U.S. Standard screen size of between No. 10 and 80. "Soy Protein Concentrates" refer to those products produced from dehulled, defatted soybeans by three basic processes: acid leaching (at about pH 4.5), extraction with alcohol (about 55-80%), and denaturing the protein with moist heat prior to extraction with water. Conditions typically used to prepare soy protein concentrates have been described by Pass [(1975) U.S. Patent No. 3,897,574; Campbell et al., (1985) in *New Protein Foods*, ed. by Altschul and Wilcke, Academic Press, Vol. 5, Chapter 10, *Seed Storage Proteins*, pp 302-338]. "Extrusion" refers to processes whereby material (grits, flour or concentrate) is passed through a jacketed auger using high pressures and temperatures as a means of altering the texture of the material. "Texturing" and "structuring" refer to extrusion processes used to modify the physical characteristics of the material. The characteristics of these processes, including thermoplastic extrusion, have been described previously [Atkinson (1970) U.S. Patent No. 3,488,770, Horan (1985) In

New Protein Foods, ed. by Altschul and Wilcke, Academic Press, Vol. 1A, Chapter 8, pp 367-414]. Moreover, conditions used during extrusion processing of complex foodstuff mixtures that include soy protein products have been described previously [Rokey (1983) *Feed Manufacturing Technology III*, 222-237; McCulloch, U.S. Patent No. 4,454,804].

Also, within the scope of this invention are food, food supplements, food bars, and beverages which have incorporated therein an isoflavonoid-containing product of the invention. The beverage can be in a liquid or in a dry powdered form.

The foods to which the isoflavonoid-containing product of the invention can be incorporated/added include almost all foods/beverages. For example, there can be mentioned meats such as ground meats, emulsified meats, marinated meats, and meats injected with an isoflavonoid-containing product of the invention; beverages such as nutritional beverages, sports beverages, protein fortified beverages, juices, milk, milk alternatives, and weight loss beverages; cheeses such as hard and soft cheeses, cream cheese, and cottage cheese; frozen desserts such as ice cream, ice milk, low fat frozen desserts, and non-dairy frozen desserts; yogurts; soups; puddings; bakery products; and salad dressings; and dips and spreads such as mayonnaise and chip dips. The isoflavonoid-containing product can be added in an amount selected to deliver a desired dose to the consumer of the food and/or beverage.

In still another aspect this invention concerns a method of producing an isoflavonoid-containing product which comprises: (a) cracking the seeds obtained from transformed plants of the invention to remove the meats from the hulls; and (b) flaking the meats obtained in step (a) to obtain the desired flake thickness.

EXAMPLES

The present invention is further defined in the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

EXAMPLE 1

Construction of Plasmids for Transformation of *Glycine max*

The ability to increase the levels of isoflavonoids in transgenic plants was tested.

For this purpose, expression cassettes were prepared that would be capable of

5 suppressing flavanone 3-hydroxylase, or that would express the CRC chimera.

Plasmids AC21 and pOY135 were prepared and the methods used to prepare them are described below.

Preparation of plasmid AC21

10 Plasmid AC21 was prepared to test the effect of the expression of a soybean flavanone 3-hydroxylase on the soybean isoflavonoid profile. To prepare plasmid AC21, expression cassettes were prepared containing a seed-specific expression promoter followed by nucleotide sequences that promote formation of a stem loop structure surrounding sequences encoding a portion of a soybean flavanone 3-hydroxylase gene, and followed by a transcription termination signal. It is well
15 understood by those skilled in the art that other sequences commonly used in molecular manipulations may be used here. These sequences may include any seed-specific promoter, any structure that promotes stem-loop formation, any portion of the gene or genes of interest inserted in sense or anti-sense orientation with respect to the promoter and stem-loop structure, and any termination signal. It
20 is also well known by those skilled in the art that sequences promoting stem-loop formation are not always required for gene suppression. Preparation of plasmid AC21 follows:

Plasmid AC21 was prepared by inserting a polynucleotide encoding a portion of a soybean flavanone 3-hydroxylase in the seed-specific expression vector
25 pKS151. Vector pKS151 is depicted in Figure 2 and its nucleotide sequence is shown in SEQ ID NO:1. This vector has been described in PCT Publication WO 02/00904, published 03 January 2002, and is derived from the commercially available vector pSP72 (Promega, Madison, WI).

To produce pKS151 vector pSP72 was modified by:

- 30 a) deleting nucleotide sequences 1135 through 1995 corresponding to the beta lactamase coding region;
- b) inserting sequences encoding HPT under the control of the T7 promoter and termination signals, for expression of the HPT enzyme in bacteria;
- c) adding a cassette comprising the 35S promoter/HPT/NOS 3' for constitutive
35 expression of the HPT enzyme in plants; and

d) adding a cassette comprising a unique Not I restriction endonuclease site surrounded by sequences that promote formation of a stem structure which are flanked by the KTi promoter and KTi 3' terminator sequences.

Expression of HPT by two different promoters allows the selection for growth in the presence of hygromycin in bacterial and plant systems. The promoter and transcription terminator from the gene encoding the kunitz trypsin inhibitor 3 (KTI3; Jofuku, K.D. and Goldberg, R.B. (1989) *Plant Cell* 1:1079-1093) in the cassette include about 2088 nucleotides upstream (5') from the translation initiation codon and about 202 nucleotides downstream (3') from the translation stop codon of KTI 3. Between the 5' and 3' regions is a unique Not I restriction endonuclease site. The Not I site is flanked by nucleotide sequences that promote formation of a "stem-loop" structure when nucleotide sequences of the gene of interest are inserted at the Not I site. This "stem-loop" structure will have the sequences of the gene of interest forming the loop. The stem structure is formed by two copies of a 36 nucleotide sequence at the 5' end of the Not I site and an inverted repeat of the same two 36-nucleotide sequences at the 3' end.

To create plasmid AC21 sequences encoding a portion of a soybean flavanone 3-hydroxylase gene were inserted in the Not I site of pKS151. The flavanone 3-hydroxylase sequences were obtained by PCR amplification using clone sfl1.pk0040.g11 as template and primers flavanone 3-hydroxylase-Not1-sense (shown in SEQ ID NO:2) and flavanone 3-hydroxylase-Not1-antisense (shown in SEQ ID NO:3). Clone sfl1.pk0040.g11 was originally identified as encoding a flavanone 3-hydroxylase in PCT publication WO 99/43825, published 02 September 1999. The nucleotide sequence of the cDNA insert in clone sfl1.pk0040.g11 is shown in SEQ ID NO:4.

5'-GCG GCC GCA TGG CAC CAA CAG CCA AG-3' (SEQ ID NO:2)

5"-GCG GCC GCA TCC GTG TGG CGC TTC AG-3' (SEQ ID NO:3)

The amplification reaction was performed using advantage 2 polymerase and GC melt reagent (1mM final concentration) and following to the manufacturer's (Clontech, Palo Alto, CA) protocol. The resulting amplified DNA fragment was first cloned into TopoTA vector (Invitrogen, Carlsbad, CA). The fragment was liberated from the TopoTA vector by Not I digestion and was purified from an agarose gel using Qiagen Gel Purification Kit (Qiagen, Valencia, CA). The purified DNA fragment was inserted into the Not I site of vector pKS151 to produce the plasmid AC21.

Preparation of plasmid pOY135

Plasmid pOY135 contains, flanked by Hind III restriction endonuclease sites, a CRC chimeric construct inserted between the phaseolin promoter and polyadenylation signal sequences. The CRC chimeric construct contains, between

5 Sma I sites and in the 5' to 3' orientation, maize nucleotide sequences encoding

a) the C1 myb domain to amino acid 125;

b) the entire coding region of the Lc allele of R (amino acids 1 through 160);

and

c) the C1 transcription activation domain (from amino acid 126 to the

10 C-terminus of C1).

The CRC chimeric construct was isolated from plasmid pDP7951 (described in PCT Publication WO 00/44090, published August 3, 2000, and bearing ATCC deposit No. PTA371) and inserted into vector pCW108N. Vector pCW108N is derived from the commercially-available vector pUC18 (Gibco-BRL) and contains

15 between Hind III sites:

a) a DNA fragment of the phaseolin gene promoter extending from -410 to +77 relative to the transcription start site (Slightom et al. (1991) *Plant Mol. Biol. Man. B16:1*); and

b) a 1175 bp DNA fragment including the polyadenylation signal sequence
20 region of the same phaseolin gene (see sequence descriptions in Doyle et al. (1986) *J. Biol. Chem.* 261:9228-9238 and Slightom et al. (1983) *Proc. Natl. Acad. Sci. USA* 80:1897-1901).

Plasmid pCW108N was digested with Asp 718, which cuts between the phaseolin promoter and polyadenylation signal sequence, and the protruding ends
25 filled-in by incubation with T4 DNA polymerase in the presence of dATP, dCTP, dGTP, and dTTP. The DNA fragment containing the CRC chimeric construct was isolated from pDP7951 by digestion with Sma I, purified by agarose gel electrophoresis, and inserted into the blunt-ended pCW108N to create plasmid pOY135.

30

EXAMPLE 2Transformation of Somatic Soybean Embryo Cultures
and Regeneration of Soybean Plants

The ability to increase the isoflavonoid levels in transgenic soybean plants was tested by co-transforming soybean somatic embryo cultures with plasmids AC21
35 and pOY135 or with plasmid AC21 alone, selecting transformants that grew in the presence of hygromycin, allowing plants to regenerate, and measuring the levels of isoflavone produced in seeds.

Soybean embryogenic suspension cultures were transformed with plasmid AC21 in conjunction with plasmid pOY135 by the method of particle gun bombardment.

5 The following stock solutions and media were used for transformation and regeneration of soybean plants (per Liter):

MS Sulfate 100x stock: 37.0 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.69 g $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.86 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0025 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.

MS Halides 100x stock: 44.0 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.083 g KI, 0.00125 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 17.0 g KH_2PO_4 , 0.62 g H_3BO_3 , 0.025 g $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 3.724 g
10 Na_2EDTA , 2.784 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$.

B5 Vitamin stock: 100.0 g *myo*-inositol, 1.0 g nicotinic acid, 1.0 g pyridoxine HCl, 10.0 g thiamine.

2,4-D stock: 10 mg/mL

SB55: 10 mL of each MS stock, 1 mL of B5 Vitamin stock, 0.8 g NH_4NO_3 ,
15 3.033 g KNO_3 , 1 mL 2,4-D stock, 0.667 g asparagine, pH 5.7.

SB103: 1 pk. Murashige & Skoog salt mixture (Gibco, Carlsbad, CA), 60 g maltose, 2 g gelrite, pH 5.7.

SB71-1: B5 salts, 1ml B5 vitamin stock, 30 g sucrose, 750 mg MgCl_2 , 2 g gelrite, pH 5.7.

20 Soybean (of the Jack variety) embryogenic suspension cultures were maintained in 35 mL SB55 liquid media on a rotary shaker (150 rpm) at 28°C with a mix of fluorescent and incandescent lights providing a 16 hour day, 8 hour night cycle. Cultures were subcultured every 2 to 3 weeks by inoculating approximately 35 mg of tissue into 35 mL of fresh liquid media.

25 Soybean embryonic suspension cultures were transformed by the method of particle gun bombardment (see Klein et al. (1987) *Nature* 327:70-73) using a DuPont Biolistic PDS1000/He instrument. Embryos were either bombarded with plasmid pAC21 or co-bombarded with plasmid AC21 and plasmid pOY135 in a 1:10 molar ratio. Transformed lines were selected on hygromycin containing medium,
30 and the presence of plasmid pAC21 or the CRC chimeric construct was determined by PCR. Transgenic plants were generated from lines positive for the desired recombinant DNA fragments.

For bombardment, 5 μL plasmid DNA (either 1 $\mu\text{g}/\mu\text{L}$ of pAC21 or a mixture of 0.5 $\mu\text{g}/\mu\text{L}$ pOY135 and 1 $\mu\text{g}/\mu\text{L}$ pAC21), 50 μL 2.5 M CaCl_2 , and 20 μL 0.1 M spermidine were added to 50 μL of a 60 mg/mL 0.6 μm gold particle suspension.
35 The particle preparation was agitated for 3 minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated gold particles were

then washed once with 400 μ L of 100% ethanol, resuspended in 40 μ L of anhydrous ethanol, and sonicated three times for 1 second each. Five μ L of the DNA-coated gold particles was then loaded on each macro carrier disk.

5 Approximately 300 to 400 mg of two-week-old suspension culture was placed in an empty 60 mm X 15 mm petri dish and the residual liquid removed from the tissue using a pipette. The tissue was placed about 3.5 inches away from the retaining screen and bombarded twice. Membrane rupture pressure was set at 1100 psi and the chamber was evacuated to -28 inches of Hg. Two plates were bombarded for each experiment and, following bombardment, the tissue was
10 divided in half, placed back into liquid media, and cultured as described above.

Eleven days after bombardment, the liquid media was exchanged with fresh SB55 media containing 50 mg/mL hygromycin. The selective media was refreshed weekly. Seven weeks post bombardment, green, transformed tissue was observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue
15 was removed and inoculated into individual flasks to generate new, clonally propagated, transformed embryogenic suspension cultures. Thus, each new line was treated as an independent transformation event. Soybean suspension cultures can be maintained as suspensions of embryos clustered in an immature developmental stage through subculture or can be regenerated into whole plants by
20 maturation and germination of individual somatic embryos.

Transformed embryogenic clusters were removed from liquid culture and placed on SB103 solid agar media containing no hormones or antibiotics. Embryos were cultured for eight weeks at 26°C with mixed fluorescent and incandescent lights on a 16 hour day, 8 hour night schedule. During this period, individual
25 embryos were removed from the clusters and analyzed at various stages of embryo development. Lines selected for hygromycin resistance were assayed by PCR for the presence of the CRC chimeric construct and/or the F3H construct contained in plasmid pAC21. The presence of the F3H construct was assayed by amplifying plant tissue using Primer 3 (shown in SEQ ID NO:5) and Primer 4 (shown in SEQ ID
30 NO:6), or using Primer 5 (shown in SEQ ID NO:7) and Primer 6 (shown in SEQ ID NO:8) and separating the amplified product by agarose gel electrophoresis. The presence of the F3H construct was confirmed by the appearance of a 763 base pair fragment when using Primer 3 and Primer 4, or a 656 base pair fragment when using Primer 5 and Primer 6. The presence of the CRC chimeric construct was
35 determined by amplifying plant tissue using Primer 1 (shown in SEQ ID NO:9) and Primer 2 (shown in SEQ ID NO:10) which, when separated by agarose gel

electrophoresis, produce a fragment not present in soybean plant tissue not having the CRC chimeric construct.

primer 3: 5' – TCC TCA GTC ACC GAT CTC CAC CC – 3' (SEQ ID NO:5)

primer 4: 5' – CGG ATA TAA TGA GCC GTA AAC A – 3' (SEQ ID NO:6)

5 primer 5: 5' – TGG ATG GAC GCA GAA GAG AGA TTT G – 3' (SEQ ID NO:7)

primer 6: 5' –CCG ATT CTC CCA ACA TTG CTT ATT C – 3' (SEQ ID NO:8)

primer 1 5' – AGG CGG AAG AAC TGC TGC AAC G – 3' (SEQ ID NO:9)

primer 2 5' –AGG TCC ATT TCG TCG CAG AGG C – 3' (SEQ ID NO:10)

Somatic embryos became suitable for germination after eight weeks and were then removed from the maturation medium and dried in empty petri dishes for 1 to 5 days. The dried embryos were then planted in SB71-1 medium where they were allowed to germinate under the same lighting and germination conditions described above. Germinated embryos were transferred to sterile soil and grown to maturity. Seeds were harvested.

15 EXAMPLE 3

Analysis of Isoflavone Levels in Seeds of Transformants Containing the F3H Construct or the F3H Construct and the CRC Chimeric Construct

The quantity of isoflavones in seeds from transgenic plants comprising the F3H construct or comprising the CRC chimeric construct and the F3H construct was assayed. Seeds were ground, the combined powder was extracted with methanol, hydrolyzed with base, and analyzed. Base hydrolysis removes the malonyl group from malonylglucoside conjugates and the three glucoside conjugates (genistin, daidzin, and glycitin) are measured. While aglycones are not measured by this method the amount of aglycones present is in such low quantities as to not affect the final results. Thus the isoflavone numbers are reported as parts per million (ppm) of glucoside conjugates in soybean. A more detailed explanation of the preparation of seed extracts and measurement of isoflavone follows.

Five to eight seeds per transformant were combined and the seeds were ground to a fine powder using a single seed grinder set to the finest setting. One gram of ground soybean seeds was extracted with 40 mL MeOH:water (80:20 v/v) in a 125-mL Erlenmeyer flask at 65°C on an orbital shaker. After shaking for 2 hours the flask was removed from the shaker and allowed to cool to room temperature. Three mL of 2N NaOH were then added and the flask was returned to an orbital shaker at room temperature for 10 min. The flask was then removed from the shaker and 1-mL glacial acetic acid was added. The sample was diluted to 50 mL with MeOH:water (80:20 v/v) and filtered through 5 µM filter paper in a funnel into another 125-mL Erlenmeyer flask. A mixture of 2.5 mL of sample and 2.5 mL of MeOH:water (80:20 v/v) were diluted with water to 10 mL in a volumetric flask.

Particulate material was removed from a sample of 1-1.5 mL by spinning in a microfuge tube, the liquid transferred to a labeled autosampler vial, and analyzed by HPLC using the gradient indicated on Table 2 below.

5

TABLE 2
HPLC Gradient Settings

Time (min)	Flow (mL/min)	1% acetic acid in water (mL)	1% acetic acid in acetonitrile (mL)
Initial	1.0	90	10
5.0	1.0	90	10
11.0	1.0	78	22
12.0	2.0	0	100
14.5	2.0	0	100
14.6	2.0	90	10
16.5	1.0	90	10

The HPLC was set to continue acquiring data to 17 minutes. Degas mode was set to continuous, column temperature was set to 30°C, and sample temperature was set to 4°C. The ultraviolet detector was set as follows: sampling rate = 10, wavelength = 262 nm, autozero = 0.1 minutes. The amounts of isoflavones present were determined by comparing the results to a 5-point standard curve conducted using commercially available daidzin, glycitin, and genistin.

Analysis of R1 Seed From Transformation Events With plasmid AC21

The levels of isoflavones in 27 independent transformation events containing plasmid AC21 alone were assayed. Table 3 and Figure 3 present the level of isoflavones (total), as well as the isoflavone components (daidzin, glycitin, or genistin), of samples of transgenic seeds positive for the F3H construct. For ease of understanding, the plants from which the seeds are derived are numbered 1 through 30 in the figure, and the plant number indicated in the table.

TABLE 3
Isoflavone and Isoflavone Component Levels
in R1 Seed Transgenic for Plasmid AC21

Plant	Plant No.	Daidzin	Glycitin	Genistin	Total
1	264-2-4-1	1019	448	1957	3424
2	264-2-4-2	1026	496	1885	3407
3	264-2-5-1	490	391	1007	1888
4	264-2-5-3	455	472	846	1774
5	264-2-6-1	737	685	2298	3720
6	264-2-6-2	845	673	1842	3360
7	264-3-3-1	412	397	820	1629
8	264-3-3-2	500	414	1070	1984

Plant	Plant No.	Daidzin	Glycitin	Genistin	Total
9	290-1-6-1	1183	480	1975	3638
10	290-1-6-3	812	327	1542	2681
11	290-2-14-2	173	215	331	719
12	290-2-23-1	323	341	629	1293
13	290-2-23-2	587	432	1061	2080
14	290-2-23-3	263	348	533	1144
15	290-2-24-1	300	285	654	1239
16	290-2-24-3	231	235	575	1041
17	262-1-4-1	237	315	532	1084
18	262-1-4-2	601	399	1109	2109
19	262-1-4-3	574	482	1131	2187
20	262-1-10-1	531	470	962	1963
21	262-1-10-3	701	490	1218	2409
22	262-2-1-1	237	243	615	1095
23	262-2-1-2	318	276	884	1478
24	262-2-7-1	223	317	470	1010
25	262-2-7-2	319	398	745	1462
26	262-2-7-3	317	356	727	1400
27	262-3-6-1	415	460	1184	2059
28	262-3-6-2	351	469	745	1565
29	262-3-8-1	253	363	577	1193
30	262-3-8-2	502	399	963	1864

The levels of isoflavone accumulation did not vary significantly in any of the transformants positive only for plasmid AC21 (F3H construct) when compared to wild type (untransformed) soybeans of the Jack variety.

5 *Analysis of R1 Seed From Transformation Events With plasmids AC21 and pOY135*

The levels of isoflavones in plants from independent transformation events containing plasmid AC21 and plasmid pOY135 were assayed. Table 4 and Figure 4 through Figure 9 present the level of isoflavones, as well as the isoflavone components, of samples of transgenic seeds positive for the F3H construct and the CRC chimeric construct. For ease of understanding, the plants from which the seeds are derived are numbered 1 through 180 in the figures, and the plant number indicated in the table.

TABLE 4
Isoflavone and Isoflavone Component Levels
in R1 Seed Transgenic for Plasmids AC21 and pOY135

Plant	Plant No.	Daidzin	Glycitin	Genistin	Total
1	275-2-2-1	157	446	320	923
2	275-2-2-2	227	517	466	1211
3	275-2-3-2	870	595	1598	3063
4	276-2-4-1	366	348	717	1431
5	276-2-4-2	304	370	618	1292
6	276-4-6-2	746	430	1293	2469
7	276-4-6-3	362	340	617	1319
8	277-4-3-1	522	480	1112	2114
9	277-4-3-2	549	334	1128	2011
10	277-4-4-1	138	351	281	770
11	277-4-4-2	382	530	842	1755
12	277-4-6-2	1203	576	1876	3655
13	277-4-6-3	498	426	907	1831
14	277-6-3-1	8684	1246	2130	12060
15	277-6-3-2	4126	919	3936	8981
16	277-6-7-1	867	506	1549	2923
17	277-6-7-3	611	546	1097	2254
18	277-7-6-1	940	434	1364	2738
19	279-2-1-1	5326	1049	627	7002
20	279-2-1-2	3308	988	770	5066
21	279-3-3-2	740	473	1384	2597
22	279-3-3-3	570	436	1013	2019
23	281-2-3-1	914	405	1683	3003
24	281-2-3-2	1136	445	1962	3543
25	281-2-3-3	1205	364	2024	3593
26	281-2-3-4	619	387	1083	2088
27	281-2-3-5	263	434	594	1291
28	281-2-3-6	658	426	997	2082
29	282-3-1-1	1083	576	1811	3469
30	282-3-3-2	202	279	485	967
31	3090-1-1-2	1463	1093	1976	4532
32	3090-1-1-3	896	560	1290	2746
33	3090-1-2-2	1200	733	1948	3882
34	3090-1-2-3	509	618	943	2070
35	3090-1-3-1	1162	849	2039	4051
36	3090-1-3-2	1464	815	2076	4354
37	3090-1-6-1	2603	1104	835	4541
38	3090-1-6-2	2156	1184	966	4305
39	3090-1-8-1	7018	878	2094	9990
40	3090-1-8-2	6950	789	1001	8739
41	3090-1-9-1	326	699	862	1888

Plant	Plant No.	Daidzin	Glycitin	Genistin	Total
42	3090-1-9-2	1007	851	1178	3036
43	3090-1-9-3	953	942	1187	3083
44	3090-1-10-1	980	582	1707	3269
45	3090-1-10-2	776	591	1741	3108
46	3090-1-12-2	656	533	1208	2397
47	3090-1-12-3	640	515	1270	2425
48	3090-1-15-1	1173	865	1726	3765
49	3090-1-15-2	821	779	1208	2808
50	3090-1-17-1	1417	909	2535	4861
51	3090-1-17-2	890	568	2743	4200
52	3090-1-18-1	1335	905	2428	4668
53	3090-1-18-2	1190	1075	2064	4329
54	3090-1-18-3	1476	1333	2592	5401
55	3090-2-2-1	3499	980	1564	6043
56	3090-2-2-2	3018	725	1515	5259
57	3090-2-3-1	2644	808	1038	4491
58	3090-2-3-2	3139	853	1196	5189
59	3090-2-4-1	635	1063	1415	3113
60	3090-2-4-2	690	637	1658	2985
61	3090-2-4-3	879	778	1502	3160
62	3090-2-5-1	7185	1202	4342	12729
63	3090-2-5-2	5184	1105	5170	11459
64	3090-2-6-1	1698	699	1864	4261
65	3090-2-6-3	2037	675	2831	5543
66	3090-2-7-1	359	715	857	1932
67	3090-2-7-2	397	582	1081	2060
68	3090-2-8-1	1173	929	2228	4330
69	3090-2-8-2	1245	973	2306	4523
70	3090-2-9-1	555	734	962	2251
71	3090-2-9-2	765	1106	1543	3414
72	3090-2-11-1	739	781	1553	3073
73	3090-2-11-2	1092	1006	1919	4017
74	3090-2-12-1	2689	591	438	3719
75	3090-2-12-2	1027	391	356	1774
76	3090-2-14-2	1324	660	752	2736
77	3090-2-15-1	1252	761	2420	4433
78	3090-2-15-2	1255	769	2239	4263
79	3090-2-15-3	2158	1462	3628	7248
80	3090-2-16-1	5673	1357	4444	11474
81	3090-2-16-2	5025	1318	4388	10730
82	3090-4-1-1	899	507	1322	2727
83	3090-4-1-3	816	452	1018	2286
84	3090-4-3-2	2147	620	1427	4194
85	3090-4-4-1	587	894	1085	2566

Plant	Plant No.	Daidzin	Glycitin	Genistin	Total
86	3090-4-4-2	394	601	920	1915
87	3090-4-6-1	254	542	430	1226
88	3090-4-6-2	245	637	350	1233
89	3090-4-7-1	2195	747	1603	4545
90	3090-4-8-1	4024	1207	684	5915
91	3090-4-8-2	3931	1317	660	5908
92	3090-4-10-1	171	486	179	836
93	3090-4-11-1	646	482	1232	2359
94	3090-4-11-3	772	569	1312	2654
95	3090-4-12-1	214	456	404	1074
96	3090-4-12-3	838	746	1437	3021
97	3090-4-13-1	576	741	1221	2538
98	3090-4-13-2	608	625	1262	2495
99	3090-4-16-1	1182	704	2080	3966
100	3090-4-16-2	1112	693	1812	3618
101	3090-4-18-1	618	532	1115	2266
102	3090-5-1-1	783	540	1404	2728
103	3090-5-1-2	849	526	1273	2648
104	3090-5-4-1	1370	553	989	2912
105	3090-5-4-2	1156	511	735	2402
106	3090-6-1-2	1920	2267	4167	8354
107	3090-6-1-3	962	900	1884	3746
108	3090-6-2-1	3840	1379	871	6091
109	3090-6-2-2	929	1009	1670	3609
110	3090-6-2-3	1032	895	1743	3670
111	3090-6-3-1	2977	1180	620	4776
112	3090-6-3-2	3119	1177	658	4953
113	3090-6-6-1	512	668	1186	2366
114	3090-6-6-2	799	655	1974	3428
115	3090-6-7-1	412	711	1252	2375
116	3090-6-7-2	511	736	1300	2546
117	3090-6-8-1	3405	533	2703	6642
118	3090-6-8-2	1529	415	3249	5193
119	3090-6-9-1	516	469	1045	2029
120	3090-6-9-3	325	502	566	1393
121	3090-7-2-1	6031	1617	5053	12701
122	3090-7-2-3	5301	1654	5102	12057
123	3090-7-3-1	293	621	560	1475
124	3090-7-3-2	843	710	1265	2818
125	3090-7-4-1	955	1006	1588	3549
126	3090-7-4-3	844	1072	1471	3387
127	3090-7-5-1	1295	729	2342	4366
128	3090-7-5-2	1190	831	2029	4050
129	3090-7-6-1	1475	1068	3994	6536

Plant	Plant No.	Daidzin	Glycitin	Genistin	Total
130	3090-7-6-2	3845	752	3449	8047
131	3090-7-7-1	765	712	1771	3247
132	3090-7-7-2	669	706	1330	2706
133	3090-7-8-1	1979	937	1985	4902
134	3090-7-10-2	536	540	1555	2631
135	3090-7-11-1	818	663	1507	2987
136	3090-7-11-2	996	601	1820	3416
137	3090-7-12-1	3528	1448	1405	6381
138	3090-7-12-2	6486	2346	814	9647
139	3090-7-14-1	318	539	784	1642
140	3090-7-14-2	321	619	822	1761
141	3090-8-1-1	5399	1030	1689	8118
142	3090-8-1-3	2332	602	422	3357
143	3090-8-5-1	815	995	1335	3145
144	3090-8-5-2	798	949	1389	3137
145	3090-8-8-2	635	500	1326	2462
146	3090-8-8-3	527	607	1307	2442
147	3090-8-10-1	423	540	1005	1968
148	3090-8-10-2	263	562	713	1538
149	3090-8-11-1	419	725	728	1871
150	3090-8-11-2	634	449	1169	2252
151	3090-8-13-1	154	374	364	892
152	3090-8-13-2	875	618	1528	3021
153	3090-8-14-1	1288	618	2433	4340
154	3090-8-14-2	1457	790	2429	4675
155	3090-8-14-3	1679	939	2421	5039
156	3090-8-15-2	2011	597	2610	5219
157	3090-8-15-3	2234	608	2891	5733
158	3092-1-4-1	684	564	1248	2496
159	3092-2-3-1	936	440	1343	2718
160	3092-2-3-2	2210	744	843	3797
161	3092-2-7-1	243	437	563	1243
162	3092-2-7-2	260	367	523	1150
163	3092-3-2-2	324	421	702	1446
164	3092-3-9-1	1622	958	1491	4072
165	3092-3-9-2	809	424	1116	2350
166	3092-3-21-1	134	318	294	746
167	3092-3-21-2	142	345	201	687
168	3092-3-21-3	144	403	389	935
169	3092-3-24-1	635	407	1327	2369
170	3092-3-24-2	723	696	1127	2546
171	3092-4-10-1	1504	929	893	3326
172	3092-4-10-2	651	658	768	2077
173	3092-5-1-1	162	472	185	819

Plant	Plant No.	Daidzin	Glycitin	Genistin	Total
174	3092-5-1-3	196	463	227	885
175	3092-5-8-1	615	881	893	2389
176	3092-5-8-2	630	852	989	2470
177	3092-8-3-1	359	501	776	1635
178	3092-8-3-2	257	549	381	1188
179	3092-8-6-1	383	740	705	1828
180	3092-8-6-2	302	701	559	1561

Analyses of these transgenic events indicated that 16 out of 180 plants containing both the CRC chimeric construct and the F3H construct showed isoflavone levels greater than 7000 ppm with the highest level being 12729 ppm.

- 5 These plants accumulate isoflavones to levels significantly higher than wild type while retaining the wild type profile of the individual isoflavone components. For example seed 277-6-3-1 showed 12060 ppm isoflavones and seed 279-2-1-1 showed 7002 ppm isoflavones while wild type (untransformed) soybeans of the Jack variety show an average of 3000 ppm isoflavones.

10 *Analysis of R2 Seeds*

- The isoflavone levels of R2 seeds derived from plants containing plasmid AC21 and plasmid pOY135 were determined. Table 5 and Figure 10 present the level of isoflavones, as well as the isoflavone components, of samples of R2 transgenic seeds derived from seeds positive for the F3H construct and the CRC chimeric construct. In the genotype column, a plus sign (+) means that the progeny is PCR positive for both CRC and F3H constructs while a minus (-) means that the progeny is PCR negative for both CRC and F3H constructs, *ND is indicated where the genotype was not determined. PCR amplification was carried out as described in Example 2. Thus the high isoflavonoid phenotype is inherited in the R2 generation. For ease of understanding, the plants from which the seeds are derived are numbered 1 through 39 in the figures, and the plant number indicated in the table.

TABLE 5

Isoflavone and Isoflavone Component Levels in R2 Seed

Derived from Transgenic plants comprising Plasmids AC21 and pOY135

Plant	R1 Plant No.	R2 No.	Genotype	Daidzin	Glycitin	Genistin	Total
1	279-2-1-1	835-1	-	242	445	494	1181
2	279-2-1-1	835-2	-	152	198	330	680
3	279-2-1-1	835-3	+	2395	1182	2712	6289
4	279-2-1-1	836-1	-	229	316	464	1008
5	279-2-1-1	836-2	+	743	470	542	1756

Plant	R1 Plant No.	R2 No.	Genotype	Daidzin	Glycitin	Genistin	Total
6	279-2-1-1	836-3	+	750	752	1503	3004
7	279-2-1-1	837-1	+	1390	693	1762	3845
8	279-2-1-1	837-2	-	143	231	281	654
9	279-2-1-1	838-1	-	348	449	669	1466
10	279-2-1-1	838-2	+	2561	1086	1201	4848
11	279-2-1-2	839-1	+	1499	777	612	2887
12	279-2-1-2	839-2	+	987	631	322	1940
13	279-2-1-2	839-3	+	1638	698	604	2940
14	279-2-1-2	840-1	-	292	362	589	1243
15	279-2-1-2	840-2	-	364	335	754	1452
16	279-2-1-2	841-1	-	153	234	359	746
17	279-2-1-2	842-1	-	319	330	671	1319
18	279-2-1-2	842-2	+	2209	921	542	3672
19	277-6-3-1	878-1	-	328	411	967	1706
20	277-6-3-1	878-2	+	2037	1260	6251	9547
21	277-6-3-1	879-1	+	1739	1336	9560	12634
22	277-6-3-1	880-1	+	1261	1070	8310	10641
23	277-6-3-1	880-2	ND*	1771	1185	6538	9495
24	277-6-3-2	881-1	+	1882	1223	9360	12466
25	277-6-3-2	881-2	+	3507	1256	5587	10350
26	277-6-3-2	882-1	+	667	757	5922	7346
27	277-6-3-2	882-2	+	638	793	7394	8825
28	277-6-3-2	883-1	+	1496	937	5048	7480
29	277-6-3-2	884-1	+	1254	1072	6231	8557
30	277-6-3-2	884-2	+	1049	815	6527	8391
31	277-6-3-2	884-3	ND*	1172	983	7714	9868
32	277-6-3-3	885-1	+	530	759	6751	8041
33	277-6-3-3	885-2	-	255	216	973	1443
34	277-6-3-3	885-3	ND*	931	963	8721	10616
35	277-6-3-3	886-1	+	1490	1067	8242	10799
36	277-6-3-3	887-1	-	263	279	791	1333
37	277-6-3-3	887-2	-	251	251	722	1224
38	277-6-3-3	887-3	ND*	1666	877	5945	8488
39	277-6-3-3	888-1	+	467	575	4439	5481

*ND, not determined.

In summary, the expression of the CRC chimeric construct in conjunction with flavanone 3-hydroxylase cosuppression results in transformants that accumulate up to four times higher amounts of isoflavones when compared to wild type soybean while retaining the wild type profile of the individual isoflavone components. The isoflavone levels seen in these transgenic plants are higher than any wild type soybean cultivar known to date.

CLAIMS

What is claimed is:

1. A method of increasing isoflavonoid production in an isoflavonoid-producing plant, the method comprising:
 - 5 a) transforming a plant with (1) a first recombinant DNA construct comprising a polynucleotide selected from the group consisting of:
 - (i) a polynucleotide encoding all or part of a flavanone 3-hydroxylase;
 - 10 (ii) a polynucleotide from 5' and/or 3' ends of an isolated nucleic acid fragment which encodes flavanone 3-hydroxylase; or
 - (iii) a polynucleotide comprising (i) and (ii) ; and (2) at least one second recombinant DNA construct comprising a polynucleotide encoding a C1 myb transcription factor and a R myc-type transcription factor; and
 - 15 b) growing the transformed plant of (a); and
 - c) evaluating the plant or plant part obtained from the transformed plant for an increased quantity of isoflavonoid in the transformed plant or plant part as compared plant or plant parts obtained from an untransformed plant.
- 20 2. The method of Claim 1 wherein the first recombinant DNA construct further comprises a promoter operably linked, in a sense orientation, to the polynucleotide encoding all or part of a flavanone 3-hydroxylase.
3. The method of Claim 1 wherein the first recombinant DNA construct further comprises a promoter operably linked, in an anti-sense orientation, to the
25 polynucleotide encoding all or part of a flavanone 3-hydroxylase.
4. The method of Claim 2 or Claim 3 wherein the first recombinant DNA construct comprises a stem-loop structure selected from the group consisting of:
 - (1) a structure wherein the loop comprises the polynucleotide nucleotide encoding all or part of a flavanone 3-hydroxylase and (2) a structure wherein the stem
30 comprises the polynucleotide nucleotide encoding all or part of a flavanone 3-hydroxylase.
5. The method of Claim 1 wherein the plant is transformed with a first recombinant DNA construct comprising a nucleotide sequence encoding all or part of a flavanone 3-hydroxylase, a second recombinant DNA construct comprising a
35 nucleotide sequence encoding a C1 myb transcription factor, and a third recombinant DNA construct comprising a nucleotide sequence encoding an R myc type factor.

6. The method of Claim 1 wherein the first recombinant DNA construct and the second recombinant DNA construct form one recombinant expression vector.

7. The method of Claim 1 wherein the first recombinant DNA construct further comprises a seed-specific promoter.

5 8. The method of Claim 1 wherein the isoflavonoid-producing plant is selected from the group consisting of soybean, clover, mung bean, lentil, hairy vetch, alfalfa, lupine, sugar beet, and snow pea.

9. An isoflavonoid-producing plant made by the method of Claim 1, Claim 2, Claim 3, Claim 4, Claim 5, Claim 6, or Claim 7 the plant having an increased
10 quantity of isoflavonoid as compared with an untransformed plant.

10 10. The isoflavonoid-producing plant of Claim 9 wherein the plant is selected from the group consisting of soybean, clover, mung bean, lentil, hairy vetch, alfalfa, lupine, sugar beet, and snow pea.

11. Seeds or plant parts of the plant of Claim 10.

15 12. An isoflavonoid-containing product high in isoflavonoids obtained from the seeds or plants parts of Claim 11.

13. The isoflavonoid-containing product of Claim 12 wherein the product is selected from the group consisting of protein isolate, protein concentrate, meal, grits, full fat and defatted flours, textured proteins, textured flours, textured
20 concentrates, and textured isolates.

14. An extracted isoflavonoid-containing product high in isoflavonoids wherein the product is extracted from the seeds or plant parts of Claim 11.

15. A food which has incorporated therein the isoflavonoid-containing product of Claim 12.

25 16. A nutritional supplement which has incorporated therein the isoflavonoid-containing product of Claim 12.

17. A food bar which has incorporated therein the isoflavonoid-containing product of Claim 12.

30 18. A beverage which has incorporated therein the isoflavonoid-containing product of Claim 12.

19. An isoflavonoid-containing protein product high in isoflavone obtained from the seeds of Claim 11 wherein the seeds are soybean seeds.

20. The isoflavonoid-containing protein product of Claim 19 wherein the isoflavonoid product is selected from the group consisting of protein isolate, protein
35 concentrate, meal, grits, full fat and defatted flours, textured proteins, textured flours, textured concentrates, textured isolates, soymilk, tofu, fermented soy products, and whole bean soy products.

21. An extracted isoflavonoid-containing soy protein product high in total isoflavonoids wherein said product is extracted from the seeds of Claim 11 wherein the seeds are soybean seeds.

22. An isoflavonoid-producing plant comprising in its genome

- 5 (1) a first recombinant DNA construct comprising a polynucleotide selected from the group consisting of:
- (i) a polynucleotide encoding all or part of a flavanone 3-hydroxylase;
- 10 (ii) a polynucleotide from 5' and/or 3' ends of an isolated nucleic acid fragment which encodes flavanone 3-hydroxylase; or
- (iii) a polynucleotide comprising (i) and (ii) ; and
- (2) at least one second recombinant DNA construct comprising a polynucleotide encoding a C1 myb transcription factor and a R myc-type transcription factor;

15 wherein the plant or plant parts obtained from the transformed plant have an increased quantity of isoflavonoid as compared to the plant or plant parts obtained from an untransformed plant.

23. The isoflavonoid-producing plant of Claim 22 wherein the plant is selected from the group consisting of soybean, clover, mung bean, lentil, hairy
20 vetch, alfalfa, lupine, sugar beet, and snow pea.

24. Seeds or plant parts of the plant of Claim 23.

25. An isoflavonoid-containing product high in isoflavonoids obtained from the seeds or plants parts of Claim 24.

26. The isoflavonoid-containing product of Claim 25 wherein the product is
25 selected from the group consisting of protein isolate, protein concentrate, meal, grits, full fat and defatted flours, textured proteins, textured flours, textured concentrates, and textured isolates.

27. An extracted isoflavonoid-containing product high in isoflavonoids wherein the product is extracted from the seeds or plant parts of Claim 24.

30 28. A food which has incorporated therein the isoflavonoid-containing product of Claim 25.

29. A nutritional supplement which has incorporated therein the isoflavonoid-containing product of Claim 25.

35 30. A food bar which has incorporated therein the isoflavonoid-containing product of Claim 25.

31. A beverage which has incorporated therein the isoflavonoid-containing product of Claim 25.

32. An isoflavone-containing protein product high in isoflavonoid obtained from the seeds of Claim 24 wherein the seeds are soybean seeds.

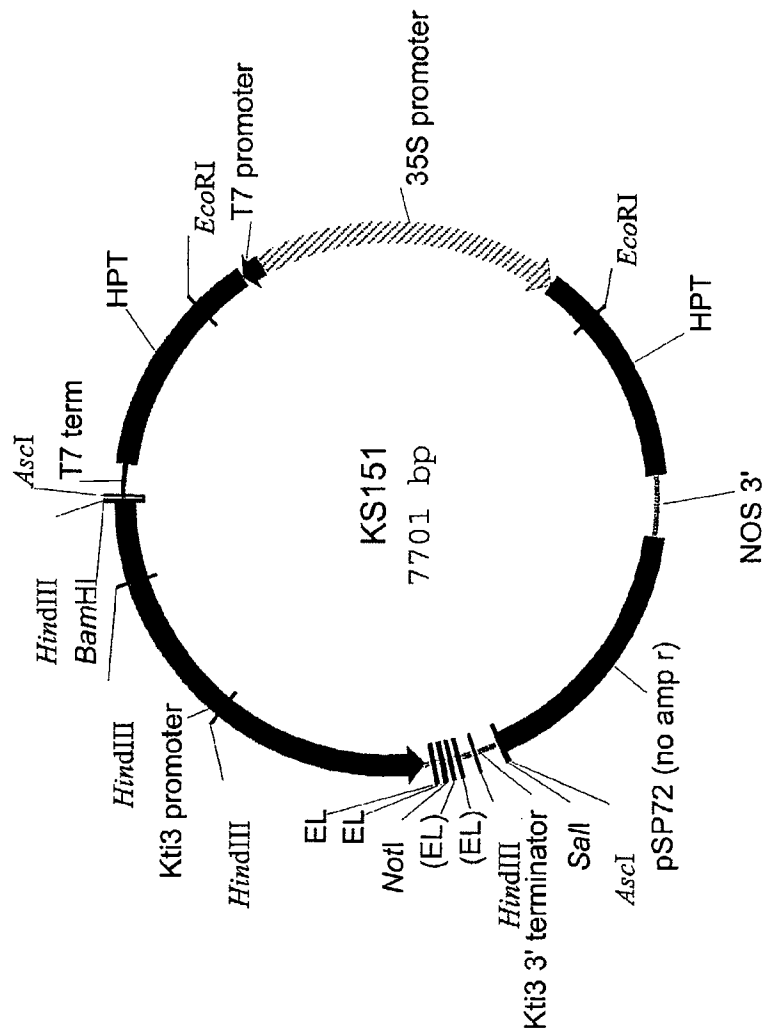
33. The isoflavonoid-containing protein product of Claim 32 wherein the isoflavonoid product is selected from the group consisting of protein isolate, protein concentrate, meal, grits, full fat and defatted flours, textured proteins, textured flours, textured concentrates, textured isolates, soymilk, tofu, fermented soy products, and whole bean soy products.

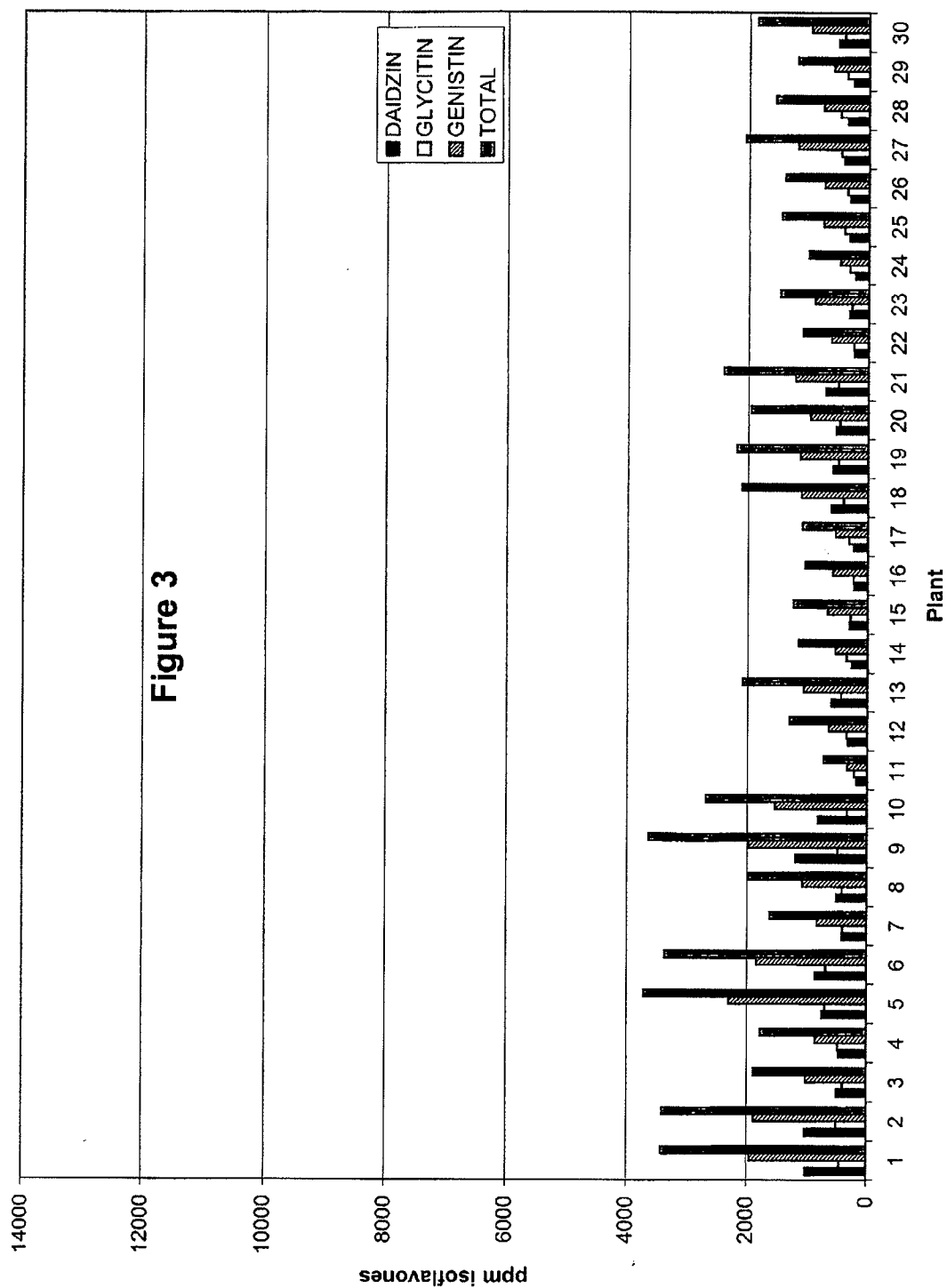
34. An extracted isoflavonoid-containing soy protein product high in total isoflavonoids wherein said product is extracted from the seeds of Claim 24 wherein the seeds are soybean seeds.

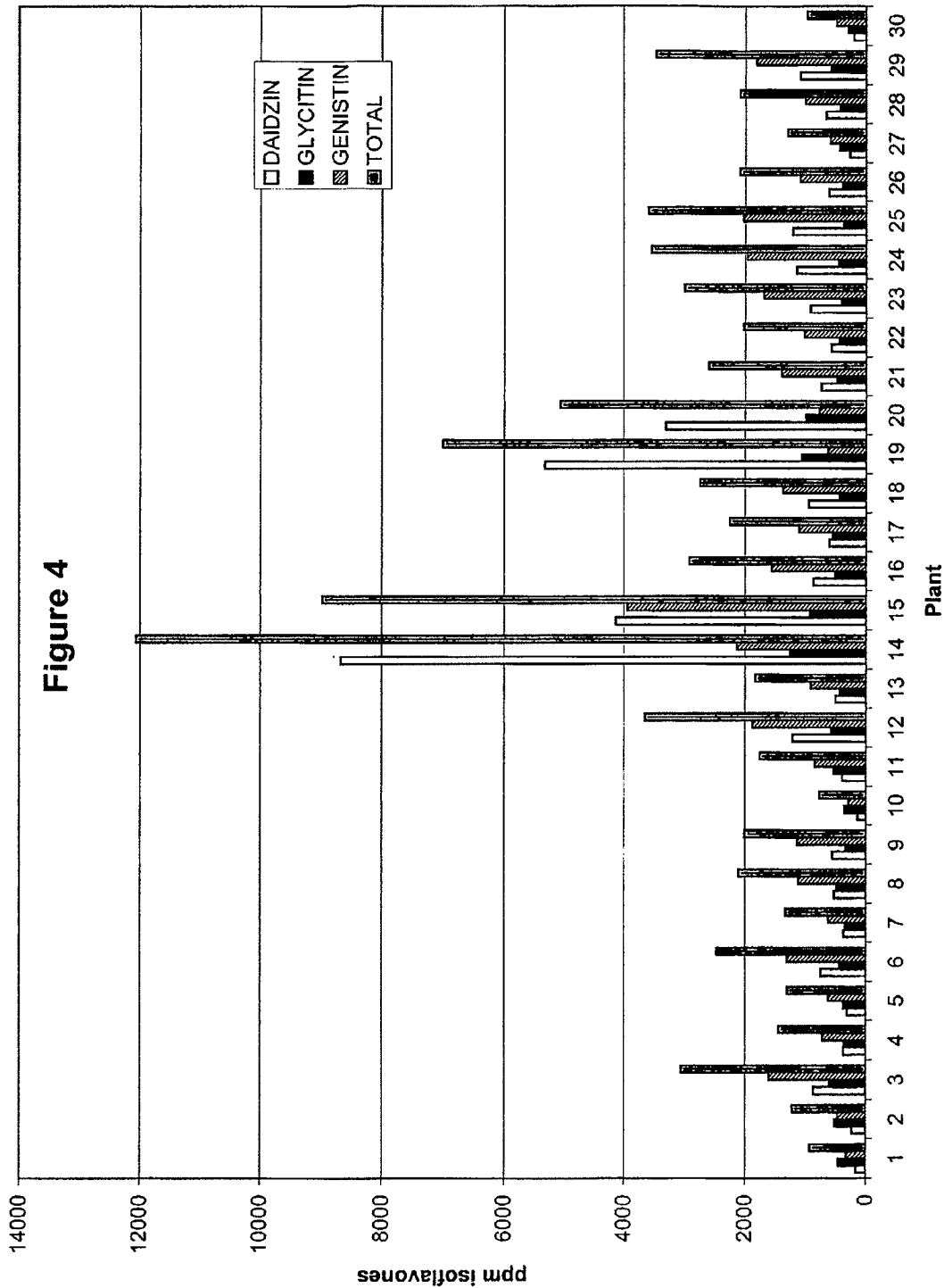
35. A method of producing an isoflavonoid-containing product which comprises:

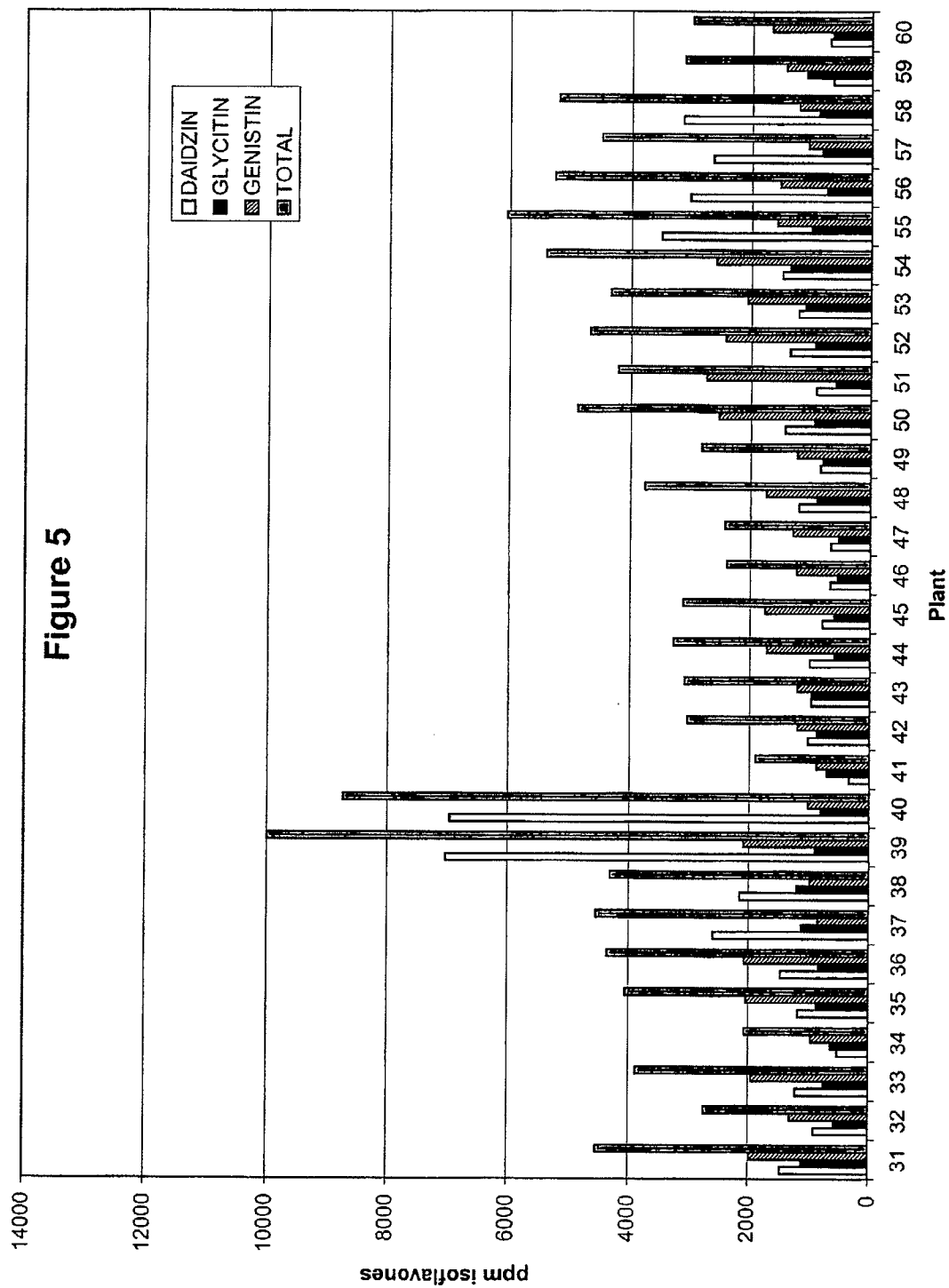
- (a) cracking the seeds of Claim 11 or Claim 23 to remove the meats from the hulls; and
- (b) flaking the meats obtained in step (a) to obtain the desired flake thickness.

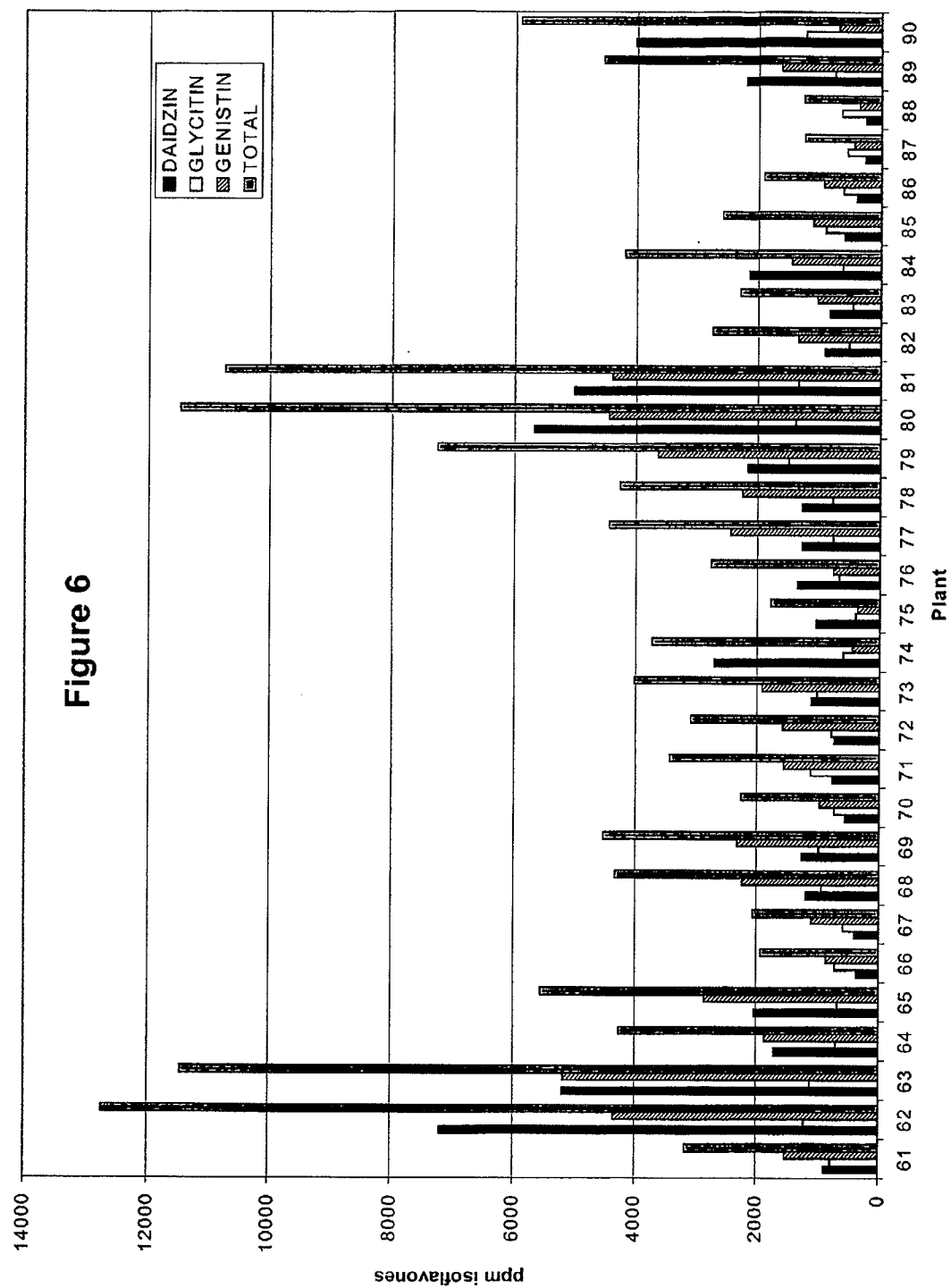
Figure 2

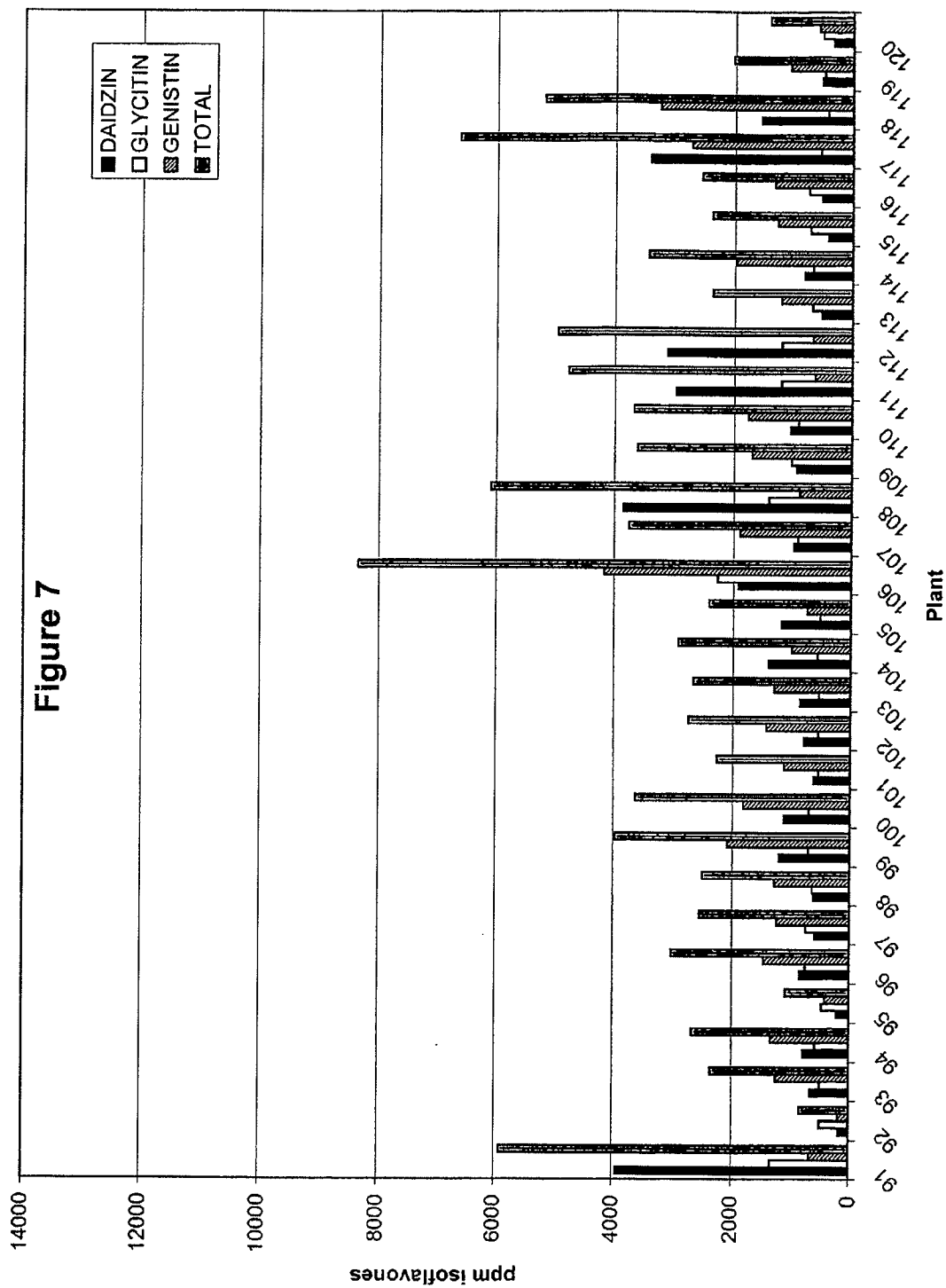


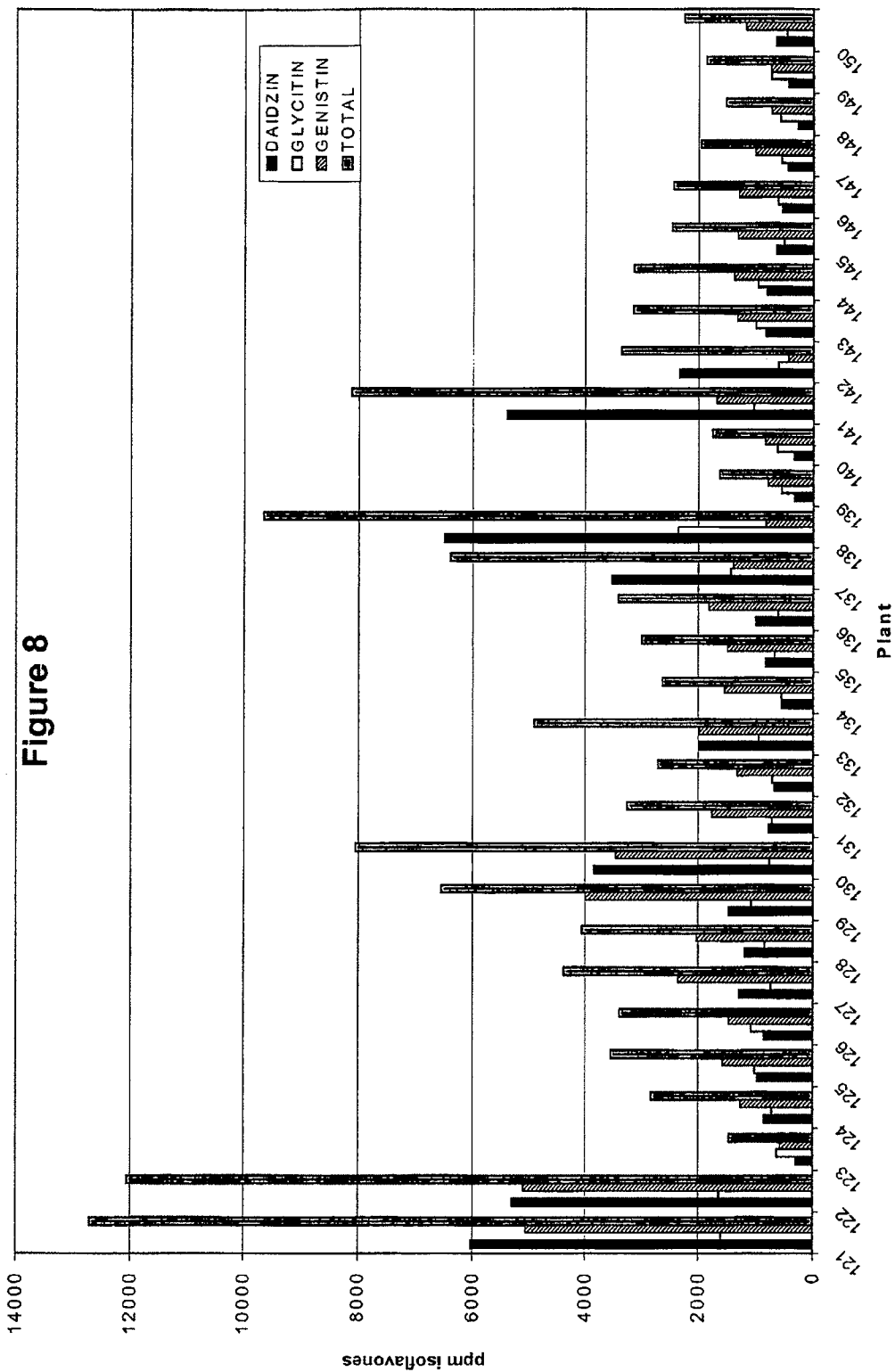


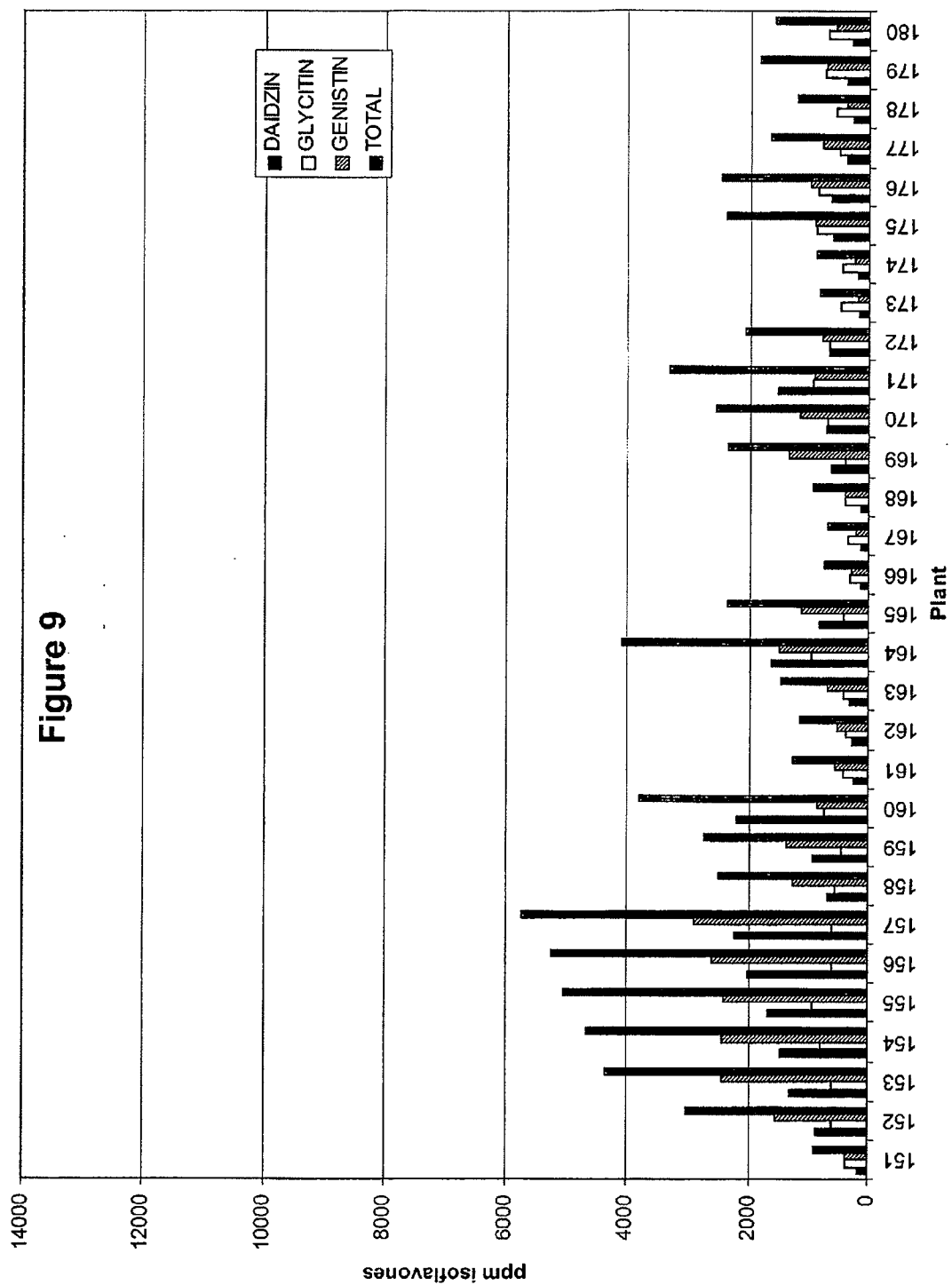


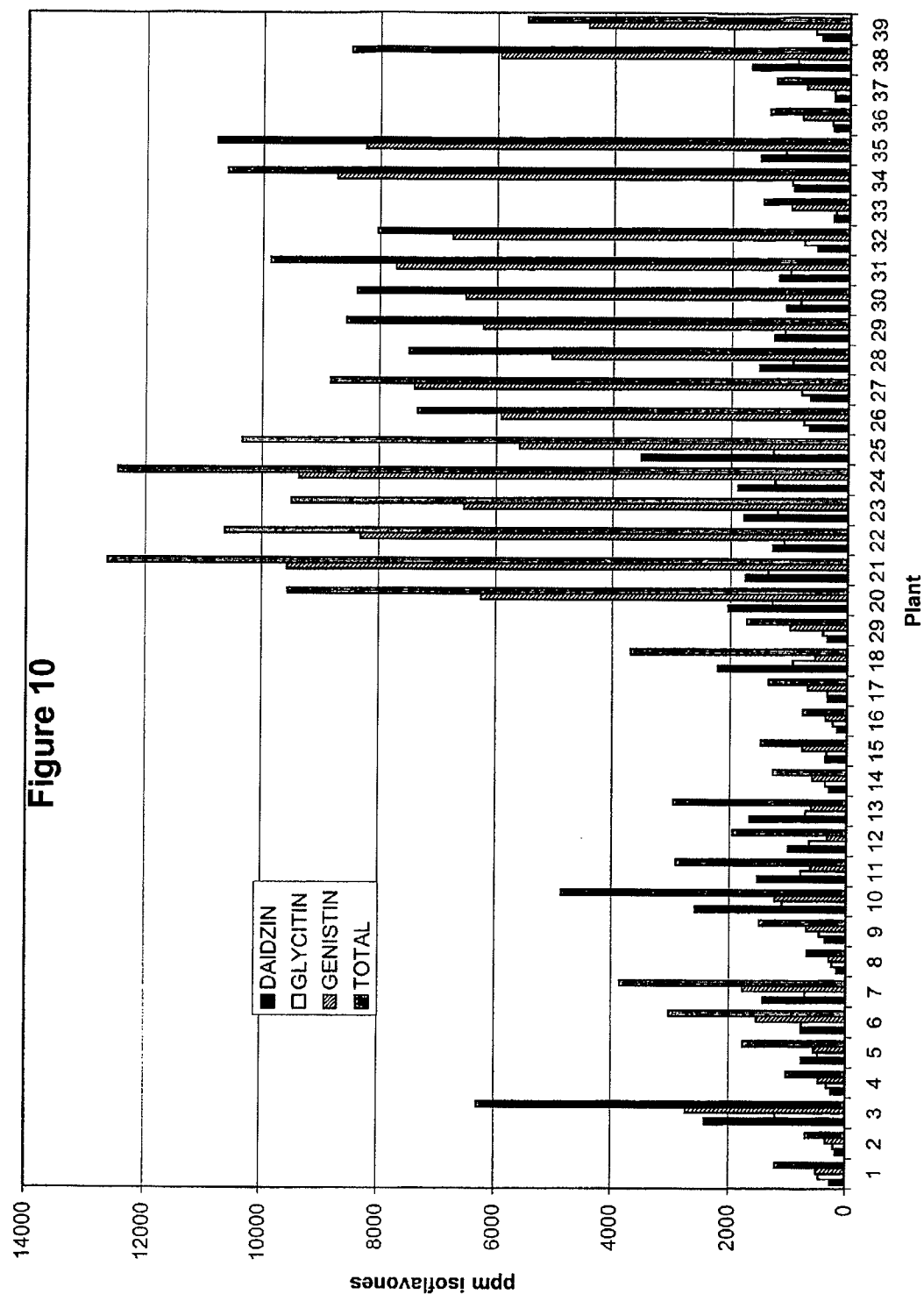












SEQUENCE LISTING

<110> E.I. du Pont de Nemours and Company

<120> METHOD TO INCREASE THE ISOFLAVONOID LEVELS IN PLANTS AND PLANTS PRODUCING INCREASED LEVELS OF ISOFLAVONOIDS

<130> BB1526 PCT

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<210> 3
<211> 26
<212> DNA
<213> Amplification primer antisense

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<400> 3
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<212> DNA
<213> Glycine max

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<210> 6
<211> 22
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<213> Amplification Primer4

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<400> 6
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<210> 7
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<213> Amplification primer5

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<210> 8
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<210> 10
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